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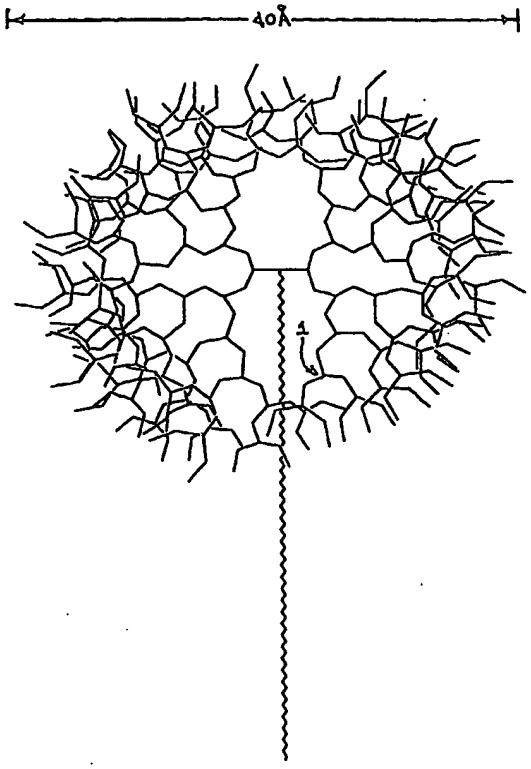
(54) Title: PHOSPHATE–BASED DENDRIMERS FOR BIOASSAYS

(57) Abstract

A new class of bioreagents is based on "closed surface" dendrimers comprising a linker arm, a forking moiety and a reporter moiety that can conjugate to a label and to a biomolecule. The bioreagents include spherical polymers where polymerization is controlled in order to achieve step–wise propagation, and thus a preselected number of reporter groups (preferably amines) on the surface. Products of this step–growth are called generations, and the number of amine groups is doubled with each subsequent generation. For the present invention the range of dendrimer generations is about 3–20, and the preferred range is 8–12.



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PHOSPHATE-BASED DENDRIMERS FOR BIOASSAYS

BACKGROUND OF THE INVENTION

This invention relates to a dendrimer, a method for its radiolabeling and applications thereof.

Biomedical applications require ever increasing sensitivity of detection and quantitation. The detection of a single organism (virus, bacteria) or even a single molecule in a few milliliters of physiological fluid is an important goal. Not only the detection, but also the quantitation of the number of organisms is important, for example in the case of HIV-1 viral load for AIDS. Often, each target should be characterized, *e.g.*, the case of metastatic cancer cells which have to be distinguished from millions of virtually identical healthy cells. Thus, in biomedical applications not only sensitivity but also specificity is of utmost importance. However, non-specific biological background (NSBB) is a limiting factor in a majority of current high sensitivity assays. New methods for signal amplification and NSBB suppression are desired which may permit a major improvement (by a factor of a hundred and more) of immunoassays and nucleic acids quantitation assays.

The development of methods for direct detection of DNA, *i.e.*, detection of DNA without the use of any amplification steps such as PCR or LCR, is enabled by the ultimate sensitivity of our Multi Photon Detection, MPDTM, technique (see U.S. Patent No. 5,532,122 the disclosure of which is incorporated herein by reference) which already permits reliable detection of a few hundreds atoms of ^{125}I and about ten atoms of the shorter life-time isotope ^{123}I. We have developed three methods for radiolabeling DNA:

* direct radiolabeling, *e.g.*, incorporation of ^{125}I-dCTP using Taq polymerase or Sequenase;
* biotinylation of DNA followed by attachment of ^{125}I- streptavidin;
* DNA probes linked to supertracer.

In the first method, ^{125}I-dCTP is incorporated with the help of appropriate enzymatic reactions. For a 100 bp duplex, about fifty cytosine sites are available. However, we have been able to radiolabel only a fraction of cytosine sites. Currently, the maximum number of ^{125I} that can be incorporated is about 10 for 100 bp duplex. Thus, currently we can detect about 100 and 10 DNA fragments with a length of a 100 bp and 1,000 bp, respectively.

When biotinylated nucleotides are used, the labeling is not limited to dCTP and up to 30 biotins can be placed on a 100 bp duplex. However, steric hindrance leads to less than

100% efficiency in binding of biotin with ^{125}I streptavidin, *i.e.*, currently similar limits of detection were achieved for direct radiolabeling and labeling using biotin as a linker. We also tried streptavidin-polyHRP, wherein polyHRP is multiply radioiodinated. For proteins, we have been able to increase the efficiency of iodination about fifty-fold. We also made a hybrid of streptavidin and a large aminodextran (MW 500,000), which has about 100 free amino groups available for attachment of ^{125}I. The use of these techniques for DNA detection will be one of the topics of study in this program. From the sensitivity point of view, with these biotin linked multi-iodinated linear constructs (b-supertracers) the detection of a few DNA fragments should be possible. The challenge is the NSBB because both polyHRP and large aminodextrans tend to be sticky.

The most promising method is the use of an appropriate DNA or PNA probe linked to a large, heavily iodinated dendrimer. A particular version of this concept has been developed by Chiron Inc. and is commercially available. Chiron uses branched DNA, *i.e.*, a linear DNA probe linked to a large and "bushy" DNA-tree. One can easily radioiodinate such "branched DNA" but the available literature suggests that in the case of tree-like dendrimers, the detection limits are not only due to sensitivity but also due to non-specific hybridization of such large DNA constructs. Thus, we opted for the development of new DNA probes linked to a "closed", nearly spherical dendrimer which promises much lower non-specific biological backgrounds.

An important aspect of the disclosed techniques is its unparalleled capabilities in detection, quantitation and analysis of proteins. In the last decades, the availability of methods for the *in vitro* amplification of DNA or RNA has revolutionized the field of biological research and has had a profound impact on the way scientific questions are being addressed. When DNA or RNA molecules are involved, amplification of the molecule of interest can be carried out *in vitro* prior to analyses and/or subsequent manipulations, e.g., cloning, sequencing, *in vitro* transcription, *etc.* As a consequence, approaches to biological questions have been considerably biased towards a genomic point of view, according to which no question can be addressed properly unless the genes involved can be cloned. However, the true actors of "life" are not genes or their transcripts, but proteins which are endowed with multiple functions: catalysis for biosynthesis and metabolism, signal transduction, bioassembly, etc. It should be stressed here that knowing the gene or being able to quantify the amounts of transcript that encode a given protein is only a part of the information. This protein may be qualitatively and quantitatively post-transcriptionally

modified in such a way that it is either active or inactive, is expressed in either large or small quantities, is either secreted or bound to the membrane, *etc.*

It would be desirable if another revolution parallel to PCR would occur in the world of protein analysis, but none is be expected in the future: alas, proteins are not self-replicating templated molecules like DNA. There is no way to amplify a protein *in vitro* other than to isolate and amplify the RNA transcript or gene that encodes it and then translates it *in vitro*. And even if this tedious procedure is to be used, which is only possible if the gene sequence is known, the copies may not be identical to the original due to post-translational modifications (*e.g.*, chaperone- aided folding, glycosylation *etc*) that occur *in vivo*, but are difficult to detect and/or to reproduce *in vitro* or in different organisms. Therefore, the fundamental issue in protein analysis is the sensitivity of the methods to detect and manipulate them. Current detection methods have reached their limits of sensitivity which explains why the scientific community has focused on the study of genes, rather than of their biologically crucial products, *i.e.*, proteins.

An aim of disclosed methods is to achieve a further few hundreds fold increase in sensitivity of MPD enabled detection methods for protein. Also, new methods of protein analysis can be improved by disclosed methods. Disclosed techniques may enable the creation of better protein databases. The improved sensitivity will allow us to follow a direct investigation methodology: isolate a protein of interest, characterize its properties (level of expression, binding to a given molecule, catalytic activity) and finally identify it. Usually, this permits the identification of gene which codes for it and mass-production of the said protein.

When proteins are to be detected, the prior art limit of detection (LOD) is typically on the order of pg/ml or 0.1 fmole/ml. Recently, with use of our MPD technique, we achieved 0.01 pg/ml or 0.5 attomole/ml sensitivity for p24 antigen (see following). Thus to detect the crucial HIV-1 protein - p24 one needs the equivalent of a few hundred viruses per sample volume. We disclose methods for NSBB elimination for microorganisms (viruses, bacteria), single cells, *e.g.*, cancer cells, and biological molecules with molecular weight larger than about 1,000 Daltons. The main focus of the invention is on nucleic acids and proteins including glycoproteins, and large lipids. We also disclose methods appropriate for the detection and quantitation of microbes and cells as well as their receptors. The aim of many of our projects is to apply the already achieved 1,000-fold increase in sensitivity of MPD enabled detection methods to the proteins and their analysis. The important step is to

correlate the genomic content of a given set of cells with the cellular functions, including the expression and secretion of rare proteins.

Immunoassays with sub-attomole/ml sensitivity may provide a much desired capacity to detect rare antigenic targets within large populations of cells. Important examples are circulating cancer cells and the initially rare HIV infected cells in individuals who will later manifest AIDS. Super-sensitive immunoassays have important implications in early diagnosis and prevention, and the control of epidemics. To overcome the NSBB we disclose modifications in the use of highly specific conjugation methods, *e.g.*, antibody-antigen or biotin-streptavidin binding. We developed a family of new reagents called SuperTracers which increase the signal as well as diminish NSBB. Techniques using appropriate solid state support(s), washing and blocking are of prior art but will be used in totally new, counter-intuitive and innovative ways in combination with other innovative steps. Some of these methods are enabled by the use of the supersensitive MPD in the step of detection.

Classical immunoassay methods. Immunoassays are a classical, reliable, specific and reasonably sensitive method for quantitation of biomolecules. There exist very specific antibodies (Ab's) to a large class of biological targets (molecules or cell surface structures). It should, however, be remembered that even the best monoclonal antibodies have a limited specificity, *i.e.*, will conjugate to a plurality of targets if they are vastly (10^9 to 10^{12} times) more abundant than the searched for antigen. A main limitation of any immunoassay is the non-specific biological background (NSBB) due to the attachment of antibodies to either the solid substrate or to other molecules in the solution. The most sensitive prior-art immunoassays (IA's) are two site IA's, often called "sandwich" assays. In this case, two different Ab's, each specific to a different antigen/epitope are used. Typically, the first Ab is used to immobilize the target organism/molecule and the second Ab is used to label the target. Depending on the physical nature of the used readout, immunoassays are defined as radioimmunoassays (RIAs), ELISA or fluoroimmunoassays (FIA), wherein radioisotopes, color labels or fluor labels are used, respectively.

An important aspect of immunoassays is that they are based on the concept of the titration curve. Thus, a relative measurement is performed instead of absolute quantitation. This permits the use of methods such as washing, blocking etc. which improve signal/background ratio but lead to unknown loss of the signal which can be calibrated using titration curves.

Using the above described methods, classical RIA, ELISA and FIA have achieved essentially the same level of performance of about 1 pg/ml for proteins, larger than say 1,000 Daltons. Thus, for a large class of macromolecules different immunoassays achieved a similar sensitivity of 0.5-5 femtomole/ml. IA's are a mature art and progress is rather slow. The limits of detection (LOD) and limits of quantitation (LOQ) are for many important biomolecules above the expected level of the molecules in physiological fluids. These LOD's and LOQ's are due to the limited specificity of classical "sandwich" immunoassays.

The IRMA is an antibody sandwich capture assay in which an immobilized antibody captures the ligand and a second radiolabeled antibody which binds to a different epitope on the ligand is used to quantitate the ligand. The amount of bound tracer antibody is directly proportional to the amount of bound ligand. IRMA sensitivity depends more on the sensitivity of the detection device and less on the binding affinity of the antibody. The sensitivity of IRMA has been limited by the backgrounds of current gamma counters (about 1 cps), and its use has been limited by the need to use hazardous quantities of radioisotope. We stress that IRMA is a much more reliable assay method than ELISA because the enzymatic signal amplification in ELISA often leads to increased inter-assay variability.

The sensitivity of current read-out methods is about 0.1 fmole/ml. Thus, only the most obvious sources of NSBB could be eliminated. Actually, the term non-specific biological background is a misnomer- it should rather be called "unknown biological backgrounds". Our studies have demonstrated that with a much more sensitive read-out system non-specific biological backgrounds can be elucidated and eliminated. However, these studies showed that there are tens of sources of NSBB, each on the level of a few attomole/ml. Only the systematic quantitation and elimination of NSBB permitted us to reach attomole/ml LOD's.

The main assumptions of classical immunoassays were analyzed and we came to the counter-intuitive conclusion that to improve specificity, *i.e.*, to increase signal/background (S/B), the signal has to be allowed to decrease considerably. This, of course, is contrary to all previous attempts to improve immunoassays including IRMA. Prior art efforts focused on maximizing the signal rather than improving the signal/background ratio. For example, in almost all prior art immunoassays washing is used. However, even when one talks about stringent washing, it is never so stringent that the signal diminishes by more than a factor of two. Blocking is also used, but once again it is never so efficient as to diminish the signal by a factor of a few. The antibody conjugation conditions are modifiable but always selected so

that at least 80% conjugation probability is achieved. All previous methods of read-out limited the sensitivity of immunoassays, and the optimal conditions were selected to improve S/B under the constraint that the signal itself is not smaller than say 20-25% of the maximum signal available. Thus, in prior art immunoassays, the S/B was not maximized but only optimized in the presence of limitations of the read-out system. MPD permits removal of these prior art limitations.

However, to allow the signal degradation, more sensitive detectors and efficient signal amplification should be implemented. We disclose reagents compatible with our MPD technique.

SuperIRMA™ Assays for Biomedical Diagnostics. A high sensitivity technique for detection and quantitative measurement of biological substances has been developed. MPD technology allows counting of specific radioisotopes at activity levels much below the natural radioactive background. It permits a reliable measurement of sub-picoCurie of ^{125}I per assay and provides 0.1 zeptomole/ml sensitivity. The technical implementations of MPD have been disclosed in our prior inventions, see, for example, U.S. Patent No. 5,532,122 relating to our MPD technique. The use of MPD permits a significant new assay protocol, the super immuno-radiometric assay, or SuperIRMA™, which provides quantitative measurement of biological substances at levels as low as a femtogram/ml. SuperIRMA is an MPD-based sandwich assay which uses biological procedures similar to the immunoradiometric assay (IRMA). We conducted studies comparing SuperIRMA to prior art immunoassay methods, *e.g.*, ELISA and IRMA. We achieved unprecedented sensitivity for a family of SuperIRMA assays for interleukin-6, p24 HIV antigen and the human gonadotropic hormones TSH, FSH, LH and hCG. Our data demonstrate the capability to develop quantitatively accurate MPD immunoassays with sensitivity below an attomole/ml using a total specific activity which is below the natural radioactive background.

We compared the sensitivity of SuperIRMA to ELISA using the immunoassay for p24, which is a core protein of the HIV-1 virion. The p24 antigen ELISA is a solid phase sandwich assay which tests for the HIV-1 virus. It is the most sensitive immunoassay currently available for HIV, and permits the measurement of viral load at early stages of infection. HIV-1 p24 antigen standard curves for the Retro-Tek™ ELISA and a SuperIRMA were compared. In the ELISA, absorbance values ranged from 0.795 down to 0.052, corresponding to p24 concentrations of 125 pg/ml to 7.8 pg/ml, respectively. In the SuperIRMA, bound radioactivity, measured with the MPD instrumentation, ranged from 146

dpm down to 1 dpm, corresponding to p24 concentrations of 30 pg/ml to 13.7 fg/ml, respectively. Since the SuperIRMA used the same reagents and protocol as the ELISA, the improved assay sensitivity could not be attributed to differences in antibody affinities or assay protocol, but rather to a more sensitive detection system. We found that the sensitivity of this commercial ELISA kit was improved about 500 fold just by using MPD technology (ELISA, 8 pg/ml *vs.* SuperIRMA, 0.014 pg/ml).

When compared with IRMA and ELISA, both SuperIRMA and immuno-PCR achieved about a 500-fold improvement in LOD's and LOQ's. Development and optimization of these assays require high cost, step-by-step recursive development procedures. The limitations of these procedures also become evident:

* for each target a *de novo* optimization process is necessary;
* the "learning curve" is very fast for LOD's at the 10-50 attomole/ml level (typically, 2-3 months) but to reach attomole/ml sensitivity a six to nine month optimization effort is necessary;
* sub-attomole/ml LOD's are only possible for certain targets;
* at the attomole/ml level, the LOD's become somewhat dependent on the quality of the antibodies;
* inter- and intra- assay variability increases below 10 attomole/ml.

Immunoassays beyond SuperIRMA . A goal of the methods disclosed in this invention is to enable the detection of a few microorganisms or cancer cells in about a milliliter of physiological fluid, which is equivalent to reaching a NSBB of zeptomole/ml of the labeled secondary antibody. Thus, further hundred-fold improvement of the sensitivity of immunoassays is necessary. However, prior art methods cannot achieve this improvement. Even the SuperIRMA is unlikely to reach limits of detection (LOD) necessary for many applications in virology, bacteriology and oncology. We believe that its main limitation is that it is but a sophisticated implementation of the "basic" sandwich assay and is limited by the specificity of any assay using one antibody to capture and another antibody to be reported.

In the majority of IA's, a single label is attached to a secondary Ab, *i.e.*, a single label is attached to either a molecule or virus/bacteria/cell. We disclose methods whereby hundreds of labels can be attached to a single protein. Another source of limitation on IA's is NSBB due to secondary Ab's spurious binding to a solid state substrate. Assuming a single labeled antibody per target, an attomole/ml LOD is roughly equivalent to the selective

detection of about 15,000 viruses/ml. However, in the case of large biological targets, *e.g.*, viruses and bacteria thousands of labeled antibodies can be conjugated to a single target. Thus, the limits of detection of the disclosed immunoassay method are a few tens of bacteria or cancer cells per ml.

Prior art immunoassays are limited to about attomole/ml sensitivity by a combination of too low a signal and limited specificity due to use of only two different antibodies. In the following, we disclose an innovative immunoassay in which a plurality, say three, four or five antibodies are used to diminish the NSBB and NSBL. Signal amplification by means of SuperTracers is disclosed. Finally, the MPD is used to permit quantitation of the labels.

By implementing an n-plet immunoassay the signal/background ratio should be considerably improved. However, the signal amplitude is diminished considerably and, for triplet assays, becomes the limiting factor. The disclosed immunoassay includes five novel features:

* use of a plurality of secondary antibodies used in parallel to increase signal;
* use of SuperTracers to amplify signal;
* use of MPD Instrumentation to quantitate signals at sub-attomole levels;
* use of "exponential" wash and other new techniques to diminish non-specific background.

More specifically, we disclose a plurality of innovative implementations of the SuperTracers which permit us to put up to thousands of radiolabels, *e.g.*, ^{125}I on a single antibody. Also, a plurality of methods to construct and use {Switch} are disclosed. The use of the supersensitive MPD technique for detection of the radiolabeled immunological reagents was disclosed in our previous Patents.

There are a plurality of applications of the proposed SuperTracers in biomedical diagnostics, namely:

* implementation of the direct quantitation of DNA/RNA methods
* improved reliability and sensitivity of "sandwich immunoassays"; and
* enabling immunoassay with a few zeptomole/ml sensitivity.

The main challenge is to implement immuno reagents which permit both increased signal and reduced non-specific biological background. The disclosed implementation is quite complicated and very counter-intuitive. It involves a plurality of innovative steps never before used in immunoassays.

The challenges f SuperTracers development. To enable the signal amplification required by the next generation of immunoassays, we designed a plurality of different SuperTracers.

In prior art assays, the label (either enzymatic, fluorescent or radioactive) is attached through the reporter group to one small molecule, and typically one or a few labels are conjugated per target molecule. In order to provide multiple labeling of the target molecule (nucleic acid or protein) in most cases long, often branched, polymers are used. A good example are branched DNA constructs labeled with fluorophores used in DNA quantitation. Another important example is the use of polyHRP in ELISA. In the case of proteins, multiple labeling usually entails use of derivatizing agents such as Bolton-Hunter reagent (N-succinimidyl-3-(4-hydroxyphenyl)propionate) to create a number of active sites for radioiodination.

When using relatively large fluorophores their number is limited by quenching. This limitation is removed when isotopic labels are used. Thus, an effective way to improve the sensitivity of an assay is to increase the specific activity of the tracer. However, steps should also be taken to reduce the biological background (nonspecific sticking). The special biological blockers and customized assay buffers developed by BioTraces can eliminate most of the biological background encountered in an assay. This makes it possible for the first time to effectively use tracers with high specific activity to generate a high signal and overcome the usual concomitant increase in biological background due to the use of large, "sticky" molecular structures.

A goal is to produce a radioactive tracer that has at least fifty times higher specific activity than ^{125}I-streptavidin which has on average less than one ^{125}I molecule per streptavidin. A few different implementations of SuperTracers with high specific activity have been developed by BioTraces. All SuperTracers employ large molecular complexes that provide multiple sites for attachment of radioisotope and functional handles for attachment to ligand. Additionally, due to special steps taken in modifying the properties of dendrimeric complexes, the recent generation of SuperTracers exhibit lower nonspecific sticking and are generally easier to remove by washing.

Implementations of SuperTracers. The first generation of SuperTracers consisted of large, essentially linear polymers appropriately radiolabeled. More specifically, we implemented radioiodinated SuperTracers using:

1. *PolyHRP - PolyHRP* is a large conglomerate of dextran/avidin/HRP which has a molecular weight in excess of 2,000,000 and which is commercially available. The multiple avidin and HRP proteins on the complex provide multiple sites for radioiodination using a standard lactoperoxidase procedure.

2. *Aminodextrans - Aminodextrans* are large molecular complexes of amino acids and dextranwhich range in molecular weight from 10,000 to 2,000,000 and which are commercially available. Free amine groups exposed on the surface of these molecular complexes can be used to attach either ^{125}I or biotin. For example, radioiodination can be performed by means of Bolton- Hunter reagent and conjugation of biotin using a succinimidyl ester biotin conjugate. We used a 500,000 MW aminodextran (Molecular Probes, Eugene, OR). The labeled aminodextrans were developed to permit bridge type reactions. Here, streptavidin served as the bridge between any kind of biotinylated detector antibody (or any biotinylated nucleic acid) and the biotinylated radioaminodextran.

3. *Polymeric derivatizing agents* - As an option to the use of biotin-avidin SuperTracers, larger polymeric multi-radioiodinated derivatizing agents can be synthesized to directly label antibodies, or any another type of detector molecule. Poly(4-hydroxystyrene) carboxy terminated chains can be synthesized to various lengths containing from 21 to 42 hydroxyphenyl groups for radioiodination. The radiolabeled polymer can then be chemically modified to produce either a carbonyl chloride or carbonyl hydrazide derivatizing agent for covalent attachment to free amine or carbonyl groups on target molecules, respectively. Preliminary experiments have been performed using this type of SuperTracer.

4. *SuperTracers using nucleic acids:* We implemented linear SuperTracers using multiply- labeled DNA or oligonucleotide. The most significant difference between the previously described and oligonucleotide-based Super Tracers implementation is diminished non-specific binding due to the weaker interaction of oligonucleotides with components of biological fluids and the surfaces of the assay containers. Also, in physiological fluids, the abundance of proteins is much higher than that of nucleic acids. In any case, all residual nucleic acids can be efficiently digested by treatment with phosphatase, which can be subsequently deactivated.

5. *Limitations of the 1st generation of SuperTracers:* The 1st generation of SuperTracers compatible with MPD used linear polymers such as radioiodinated polyHRP and aminodextrans. Actually, because ^{125}I is much smaller than typical fluorophores, more

than a hundred ^{125}I can be conjugated to a single SuperTracer molecule containing on linker to biotin moieties present on Ab. This has been demonstrated by dilution studies. It can be seen that the MPD sensitivity using ^{125}I- poly HRP is about a hundred times higher than when using singly iodinated antibodies to TSH. Notable also was the excellent linearity over five logs. However, when we developed an immunoassay using heavily iodinated streptavidin-polyHRP, the limit of sensitivity was only slightly better than when using ^{125}I-streptavidin. The disappointing result is that both the polyHRP and aminodextrans are very sticky, which leads to a high non-specific biological background. Extensive efforts to develop more efficient blocking and washing procedures failed. Thus, the NSBB appears to be an important limitation for immunoassays using these two implementations of 1st generation SuperTracers. However, the use of radiolabeled DNA as SuperTracer still seems very promising.

With the 1st generation SuperTracers, neither the binding efficiency nor NSBB were good enough to achieve sub-attomole immunoassays. However, the use of polyHRP or aminodextran based SuperTracers may be important when lower sensitivity, say at the 50 attomole/ml level, but very fast immunoassays are needed.

2nd generation SuperTracers. There are two important disadvantages of 1st generation SuperTracers. First, the number of labels per polymer is a highly variable function of the iodination conditions. Due to steric hindrance, each polymer molecule has a different number of labels. Only when hundreds of polymers are averaged is a well defined mean value obtained. An even more important disadvantage of the commonly used large linear polymers with multiple labels is their non-specific affinity to biological fluid components and surfaces of the containers used in biological assays. This problem can be easily overlooked in experiments at the femtomole/ml level which are marginally improved by increased signal, *e.g.*, in ELISAs using polyHRP. However, in super sensitive immunoassays individual elements of non-specific background should be traced and eliminated. In order to circumvent these problems, the present invention discloses a new class of SuperTracers based on "closed surface" dendrimers which are spherical polymers where polymerization is controlled in order to achieve step-wise propagation, and thus a preselected number of reporter groups (preferably amines) on the surface. Products of this step-growth are called generations, and the number of amine groups is doubled with each subsequent generation. For the present invention the possible range of dendrimer generations is 3-20, and preferred range is 8-12. The highly branched structure of

dendrimers in the latter range causes steric hindrance and forces an almost ideally spherical structure. In a preferred embodiment, the selected dendrimer is a Starburst Polyethyleneimine (PEI) dendrimer.

The 2nd generation of SuperTracers use starburst dendrimers for radioactive labeling and derivatization of the target molecule. Advantages of this approach are as follows:

* The target molecule is labeled with dendrimers bearing multiple labels.
* Due to their spherical structure dendrimers have the smallest possible surface and non-specific binding is minimal in comparison with linear polymers. For example, an 8th generation dendrimer has diameter of only 97 Angstroms, and 1,024 amino groups on its surface.
* Due to their size and weight dendrimers provide a preferred target for stringent washing.

We demonstrated that dendrimers permit better elimination of non-specific binding by promoting more effective removal of weakly bound counterparts. However, antigen-antibody complexes survive even very stringent washing.

Four distinctly different types of 2nd generation SuperTracer are made from the same radiolabeled dendrimer, followed by conjugation with one of four different functional groups.

Type 1 is conjugated with biotin using Pierce's EZ-Link NHS-LC-LC-Biotin (Succinimidyl-6'-(biotinamido)-6-hexanamido hexanoate). This type can be used as is to directly bind to streptavidin or avidin conjugates. It also binds to biotin-DNA, biotin-antibody or other biotin-protein conjugates, provided that streptavidin is used as a bridge. The binding can be enhanced by first coating the biotin-SuperTracer with streptavidin before binding to biotin-conjugates.

Type 2 is conjugated with protected SH groups using SATA (N-Succinimidyl S-Acetylthioacetate). This SuperTracer binds to maleimide-protein conjugates. For example, maleimide-streptavidin can be used to form an alternative type of Avidin-SuperTracer complex. The problem with SH groups is that if oxidized they can form disulfide bonds with other SH groups. This kind of cross-linking should be avoided, and we demonstrated that it can be diminished by using protected SH groups which can be easily unprotected before binding to biotin.

Type 3 is conjugated with maleimide groups using Sulfo-SMCC (Sulfosuccinimidyl 4-(N- maleimidomethyl) cyclohexane-1-caboxylate). This SuperTracer will bind to reduced

SH groups on proteins. It permits conjugation of antibodies to a SuperTracer. The different antibody subunits are held in place by disulfide bonds. Using gentle reducing conditions, such as mercaptolethylamine in the presence of EDTA, an antibody is split into mirror halves with exposed SH groups for conjugation with maleimide-SuperTracer. The antibody-SuperTracer construct is essential for ultrasensitive immunochromatography.

Type 4 is conjugated with a DNA probe consisting of a double stranded DNA ending with a single strand of DNA. Optionally, we use a PNA linker to increase the specificity of the binding to single stranded target DNA.

The process of production of these SuperTracers requires three consecutive operations, each using the amino moities on the surface of a starburst dendrimer:

* radiolabeling;
* linker conjugation;
* ST passivation, *i.e.,* attachment of a large number of polyglycol (PGE) molecules which diminish the nonspecific background.

In optimizing the parameters of each of these processes, however, the need to perform all three on the same object led to a plurality of trade-offs. For example, up to two hundred ^{125}I have been easily attached to the star-burst dendrimer, but full saturation, *i.e.,* to attach more than thousand ^{125}I, has not yet been achieved. Also, the attachment of linkers is a difficult step, often the avidity of attached biotin is not very good.

Thus in the following, we disclose a 3rd generation of Super Tracers, a new *ad ovo* method of production of radiolabeled SuperTracers, wherein the linker and the radiolabeling are integral part of the production process and not an *a posteriori* modification. We believe, that the disclosed process provides more versatility and allows for production of dendrimers which are

* more heavily radiolabeled (up to a few thousand ^{125}I/dendrimer);
* have better avidity for biologically important targets;
* shows lower nonspecific biological background.

SUMMARY OF THE INVENTION

The present invention comprises fully engineered, phosphate-based dendrimers, that contain only one linker group, and a strictly controlled number of reporter groups. The dendrimer is composed of several modules that can be engineered and modified according to specific needs of the final application.

1. Linker: The Linker arm is a chain that provides appropriate separation of the dendrimer from the biopolymer such as nucleic acid, protein, peptide, lipid and the like, to avoid steric hindrance during bindging. Preferably the linker arm contains carbon atoms with the most preferred embodiment being composed of repeating units of tetraethylene glycol phosphate, that provides appropriate hydrophilicity. Additionally, tetraethylene glycol phosphate can be used as a building unit such as w-O-(4,4'-dimethoxytriphenylmethyl)-1-O-(2-cyanoethyl)-N,N-diisopropyl tetraethylene glycol phosphoramidite (Fig. 1) in phosphoramidite method providing well defined and strictly controlled length of the linker.

2. Dendrimer: The dendrimer is composed of units that provide an increase of the number of the functionalities available for the chemistry in the next step. In the preferred embodiment of the present invention, dendrimer is built from glycerol-2-phosphate functionalities, where step-wise synthesis occurs on the linker arm and each coupling step results in double amount of functional groups available in the next chemical transformation. In the most preferred implementation the dendrimer is synthesized using phosphoramidite method and (1,3-O,O-bis(4,4'-dimethoxytriphenylmethyl)-2-O-(2-cyanoethyl)-N,N-diisopropylglycerolphosphoramidite (Fig.) as a building block. In this implementation functional groups are of hydroxyl type. Throughout the text the expression "generation" will be used when referring to the number of functional groups. The following definition of the generation will hence be used:

dendrimer[1] - 1st generation - first coupling resulting in 2 functional groups;

dendrimer[2] - 2nd generation - second coupling resulting in 4 functional groups;

dendrimer[10] - 10th generation - tenth coupling resulting in 1024 functional groups *etc.*

It is understood that the dendrimer building steps can be alternated with additional linker building steps resulting in formation of many dendrimers on an original dendrimer core (mega-dendrimer) as exemplified in Fig. 4 where four dendrimers are built on original 2nd generation core. This higher order structure will be referred to as "dimension". That is, there are 2^x branches in the first dimension and 2^y branches in the second dimension or 2^{x+y} total branches. For example, a two-dimensional dendrimer as in Figure 9 is called dendrimer [2,5] where the overall number of functional groups is 2^2 in the first dimension and 2^5 in the second dimension, so that 4 x 32 = 128 branches. It is also understood that there is no limit to either number of generations nor the number of generations other than steric consideration impeding the chemistry. Preferably, y is between 4 and 7.

3. Reporter groups: After full assembly of the dendrimer to required dimension and generation functional groups are transformed into reporter groups. Reporter group is a chemical moiety that is required for further derivatization and can be one of but not limited to amine, sulfhydryl, maleimide, active ester such as succinimidyl, carboxyl, allyl, alkyl, aryl, acyl, halogenoalkyl, halogenoaryl, phosphoryl, phosphorothioyl, alkyl or aryl disulfide, nucleosidyl, oligonucleotidyl, peptidyl, and the like. Choice of the reporter group is based on specific implementation of the dendrimer. It is understood that should the hydroxyl be required, the functional group becomes reporter group.

4. Terminus: Terminus is a label to be used in further implementation. Introduction of the terminus into the dendrimer can be achieved with or without the reporter group. Examples of the terminus functionalities are:

* biotin;
* radiolabels such as Bolton-Hunter reagent and its [^{125}I]iodinated derivative; ^{32}P phosphate; ^{35}S phosphorothioate;
* chelated metals such as Eu^{+3};
* antigens such as digoxigenine or bromouracyl;
* fluorophores such as fluorescein, acridine,
* conjugated enzymes or factors such as phosphatase or NAD, and the like.

It is important to emphasize that at any given point of the dendrimer construction assymetric reagent can be used in order to provide assymetric forking where one of the branches is transformed into a reporter group, and the other is used for further dendrimer generations. In this case the resulting dendrimer has reporter groups, and alternatively the labels, buried under subsequent layers of the dendrimer. This feature can be important when non-specific biological binding is major concern and the surface of the dendrimer can be used for "passivation" with reagents known to provide least non-specific binding.

5. Linker arm terminus: The linker arm terminus is the moiety that is generated after appropriate cleavage from the solid support and deprotection of the dendrimer on the end of the linker opposite to the dendrimer. In one of the preferred implementation the linker arm terminus is covalently attached to the oligonucleotide. In this case oligonucleotide is assembled first on the solid support and the appropriate modules are chemically attached to the 5' end of the oligonucleotide. The linker arm terminus can be derivatized according to known chemical methods such as use of amine generating supports.

Appropriate chemistry can be used to obtain similar variety of chemical moieties as described above for reporter groups.

BRIEF DESCRIPTION OF THE DRAWINGS

Further advantages and features will become apparent from the detailed description below taken with the drawings in which:

Figure 1 is a schematic structural representation of a bioreagent according to the invention and which is an example of the 4th generation having 16 reporter groups R_2 and linker arm terminal group R_1 , for example, an affinity group or reactive group

Figure 2 is a schematic structural representation of tetraethylene glycol repeating units of a linker arm moiety;

Figure 3 is a schematic structural representation of a forking moiety or unit in a polymer;

Figure 4 is a schematic structural representation of a bioreagent which is a SuperTracer according to the invention and which is an example of the dendrimer [2,2], *i.e.*, two dimensions and two generations each, with 16 reporter groups R_2;

Figure 5 is a schematic flow diagram of a method for preparing dendrimers according to the invention using phosphoramidite as the forking material;

Figure 6 is a schematic structural representation of a phosphoramidite - tetraethylene glycol monomer for producing a linker arm polymer;

Figure 7 is a schematic structural representation of an embodiment where R_1 is an oligonucleotide;

Figure 8 is a schematic structural representation of a branching monomer reagent to provide the branching unit of Figure 3;

Figure 9 is a schematic structural representation of a bioreagent which is a SuperTracer according to the invention;

Figure 10 is a schematic structural representation of a substrate for making amino end groups for functionalizing;

Figure 11 schematically illustrates a reaction between a Bolton-Hunter reagent and the amino terminal groups of the forking unit chains to provide an iodinated terminal reporting group for use in the method of the invention; and Figure 12 is a schematic structural representation of a substrate for asymmetric forking (DMT-levulinyl glycol phosphoramidite.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In describing preferred embodiments of the present invention illustrated in the drawings, specific terminology is employed for the sake of clarity. However, the invention is not intended to be limited to the specific terminology so selected, and it is to be understood that each specific element includes all technical equivalents which operate in a similar manner to accomplish a similar purpose.

Starburst dendrimers rely on cationic alkylamine chemistry. As described in Serial No. 60/065,063 filed November 10, 1997, incorporated herein by reference, with a starburst dendrimer, there are about 1000 active amino groups. One can iodinate up to 100-200 of them. One can passivate by attaching a "shell" PEG with chain length of 2000-3000 having low specific binding. The ratio of affinity probe to starburst dendrimer is statistical, not one-to-one or stoichiometric.

The inventive dendrimers relies instead on anionic phosphate chemistry. Prior use of phosporamidite chemistry has been limited to single forked moieties bearing two labels, or with linear chains with one arm of each fork being labeled, the other forming a chain. In contrast, according to the invention, a highly branched three dimensional dendrimer is formed. One can iodinate the dendrimer inside and outside the branched spherical structure, and can passivate the surface. The ratio of dendrimers to affinity group probes is stoichiometric, typically one to one.

The dendrimers are useful in any type of bioassay, including DNA, immunoassay, protein-ligand interactions, and so on. The dendrimers provide extraordinary signal amplification. For a kit of dendrimers, a library of oligonucleotide sequences or other terminal active groups may be prepared and used to select the appropriate analyte of interest.

The method of preparing the dendrimers is advantageous. It can be anhydrous, and handling of the solid supports is easy. The dendrimers may be constructed, dried, stored as a powder, and used when needed. The appropriate number may be counted or estimated microscopically by counting beads for precise quantitative chemistry.

The inventive dendrimer has several advantages. It increases signal where a signal is weak, for example, a single copy or a few copies of DNA. Signal amplification allows fast measurement.

The inventive dendrimer also diminishes non-specific background because it permits exponential washing as discussed in U.S.S.N 60/065,065, filed November 10, 1997, incorporated herein by reference. "Exponential" or stringent as it refers to washing

conditions is a term of art understood by those of ordinary skill to refer to those conditions of dilution, turbulence, forcefulness, temperature, chaotrophic acidity, buffer and ionic strength which permit association of a particular molecule with a conjugate molecule for which it has an intended binding affinity, while substantially inhibiting the association of molecules which have no intended binding affinity. Washing may be done under aggressive physical conditions such as ultrasonic, shade wave, vortex, agitation or by using streams of medium. The exact conditions which constitute "stringent" conditions depend on the nature and strength of the association of the molecules of interest, *i.e.*, the association constant. By varying wash conditions from a level of stringency at which non-specific association occurs to a level at which only specific association occurs, one of ordinary skill in the art can, without undue experimentation, determine conditions which will allow a given molecule to associate only with its target binding partner at levels compatible with assay sensitivity. For the present invention, because the disclosed assays are capable of attomole sensitivity, stringency may be maximized to result in a dramatic reduction of background signal while still allowing sufficient assay-specific signal for detection and quantitation.

In general a stringent wash step according to the invention is directed at reducing background at least about twice as effectively as reducing signal. Signal may be reduced below half of the original amount if background is reduced to less than a quarter and so on. In a preferred embodiment of the washing step, signal is no more than about 25% of the preceding level and background is less than about 10%. The signal may be reduced to about 20% or less by washing, while background may be reduced to less than about 1%. Appropriate conditions may be determined for each specific interaction using a standard control and a level of experimentation that is not considered to be undue by those of skill in the art.

However, it is not easy to implement such washing because it requires high enough forces to break non-specific binding without breaking specific binding. In a spherical dendrimer, the washing force is proportional to the radius of the surface. In contrast, a long linear polymer will essentially flap around or stretch, and the efficiency of washing is less. Also with a long linear dendrimer the ratio of surface to label is high, which increases the potential of non-specific binding. According to the invention a linker is long enough to reduce steric inference between the probe and its target at one end, and the spherical forking unit with reporter groups at the other.

Instead of taking an existing ball structure and modifying it, the inventive structure is built from the probe, from scratch. The chemistry is well known and has high coupling efficiencies in the range of 99.7%. It works well with solid supports and provides modules that can be arranged in many ways. If a nucleic acid probe is attached to the dendrimer, it can be made in the same equipment as the linker and forking unit, as in Figure 5. Each dendrimer is made one by one, and when the synthesis is finished, it is cleaved from the CPG glass balls.

According to the inventive method, phosphoramidite chemistry and equipment may be adapted from automatic DNA synthesis to make the inventive dendrimers, using phosphoramidite reagents instead of nucleosides. See Figure 5. A nucleotide is constructed or attached to a solid support and then it is reacted with the phosporamidite linker monomer of Figure 6. Detritylation removes the protecting groups on the reactive group, as in Figure 5. DMT is a protecting group (dimethoxytriphenymethyl or dimethoxytrityl). A hydroxyl group of the nucleotide in the general method reacts with phosphoramidite attacking the phosphorus with release of amine and then the chain is prolonged or elongated by one building unit, as shown in Figure 5 and 7. The whole cycle is repeated with oxidation and other technical transformations, each cycle adding new building blocks.

An example would be attaching dendrimer to the DNA and here there are several advantageous elements (modules) that can be used. The linker is a tail that provides some space between the oligonucleotide and forking unit dendrimer. The spacer may be a polymer made of tetraethylene glycol phosphoramidite monomers, as shown in Figures 2 and 7. The monomers are shown in 6. Tetraeethylene glycol is protected at one end with a compatible group such as dimethoxytrityl. This is reacted with DNA on a solid support and gives the same reaction as with normal coupling. To provide a linker tail of a given length one can cycle as many times as desired to form a chain of the monomers. we provide space between dendrimer and the oligonucleotide/target complex when used to bind to an analyte of interest, in this case DNA.

In Figure 8, a forking monomer is shown, and it is shown as a unit of a polymer in Figure 3. This reagent, after coupling, oxidation, and deprotection, provides a doubling of active hydroxyl groups, as shown in Figure 3. Instead of extending chain length, each coupling using the forking monomer doubles the terminal ends to provide forking. Figure 9 shows a resultant dendrimer after six levels of doubling of the dendrimer, having 2^6 or 64 branches.

After building required generations with preset numbers of hydroxyl groups, hydroxyl groups are converted into amino groups which are the most compatible with all conjugation reactions on macromolecules. 90% of derivatising agents are directed to amino group and again this can be easily done as with Figure 10. Figure 10 shows a protected substrate for providing amino groups. It is an amino spacer, a derivative of hexamethylene amino hexanol protected with monomethoxytrityl (MMT). MMT is more stable with amino groups than dimethoxytrityl. This reagent is reacted with as many as all hydroxyl groups on the forking unit dendrimer to convert them into amino groups. Then the amino groups can be efficiently and easily iodinated *e.g.* with Bolton Hunter reagent or otherwise. As shown in Figure 11, the Bolton Hunter reagent may be mono- or di-iodinated (as shown with square brackets). Also, the reagent optionally contains a sulfate group to make the reagent soluble in water. Without the sulfate group, the reagent is more soluble in organic solvents. This is a simplest example of building a multi-labeled dendrimer.

An advantage of the phosphoramidite chemistry with DMT protecting groups is that for each coupling cycle, when this group is removed, it provides a colorimetric assay that shows the efficiency of the cycle. The residue of DMT is red in the solution and can be measured by conventional assays to confirm that, up to iodination, the dendrimer is properly constructed. After each step, if saturation was not achieved, further treatment such as passivating is possible.

Preferably, there is one analytical probe, one tail, one ball. However, in other embodiments, with hydroxyl groups from the phosphoramidite moieties, one can add more linker tails and build another ball on each of them. This is referred to as "dimension" in the pattern. Dimensionality can be started earlier, for example with 8 hydroxyl groups and then 8 balls of sixth generation each can be formed as new dendrimers. Figure 4 shows a skeletal model of the beginning of a four ball dendrimer. According to computer modeling of the forking unit, the diameter of each ball is about 40 Angstroms, as shown in Figure 9.

In assymetric embodiments, selective deprotection permits use of one arm for iodination, and the other arm for the next generation. Figure 12 shows an asymmetric reagent that can be used with one of the two arms selectively protected. Then one of the arms can be used for conversion into amino group and iodination and the other arm can be used for growing next generations. For example, in Figure 9, at the point marked "1" one can stop growth and after synthesis, deprotect and iodinate. In this case, iodination will be buried under the subsequent layers of the forking reagent and this can be done at any

preselected generation. This provides more reporter groups, avoiding the following problem. Non-specific background is typically surface proportional, but surface-bound signal is also limited by surface area. With sub-surface iodination, one can increase the ratio of signal to background. Also, with spherical structures as with the invention, the surface can be passivated, *i.e.*, coated with polyethylene glycol or something else that provides very little interaction with biological systems like proteins or DNA or cells.

With sub-surface reporter groups, there are generally no pores or holes because subsequent working generations fill them. Spaces that are not filled will be filled with water and small molecules in solution. Biological molecules would not penetrate. Due to ionic groups, the dendrimer is a type of hydrogel or ionic polymer, similar in some respects to DNA.

On the outside of a forking unit one can put various labels other than or in addition to iodine. Other halogens may be appropriate, or other reporter groups. One can do chemical coupling of an oxide or oligonucleotide which could be for example enzymatically phosphorylated with a radioisotope. One can put biotin on the surface and label using the enzyme streptavidin due to the affinity between them. Streptavidin is available as a labeled molecule. This allows adding almost any functional group.

Thus, the linker arm terminal group may be any affinity group or reactive group, such as those described in U.S.S.N. 08/679,671, filed July 12, 1996, incorporated herein by reference. The reporter group may be any radiolabel for MPD or other radiodetection, or it may be another reporter group for *e.g.* enzymatic, immunoassay, fluorescence, or so on. The common elements remain the linker arm and spherical forking unit or units.

Preferred implementation of the proposed phosphate-based dendrimer is schematically shown on Figure 1, where elements of the proposed structure are shown after deprotection and cleavage from the solid support. In this example the terminus groups are not attached yet. The synthesis is preferably performed on the solid support and using phosphoramidite method analogous to the one used in the oligonucleotide chemistry. After deprotection of the support the linker reagent is added (w-O-(4,4'-dimethoxytriphenylmethyl)-1-O-(2-cyanoethyl)-N,N-diisopropyl tetraethylene glycol phosphoramidite, Figures 2 and 6) in the presence of tetrazole. Subsequent cycle steps (capping and oxidation, see Fig. 5.) give O- protected linker arm with a phosphate group and tetraethylene glycol. Should the extension of the arm be necessary, the cycle can be repeated as many times as practical, where each coupling adds about 18_ of the spacer arm. When

the spacer arm is constructed, in the next cycles branching phosphoramidite is used (1,3-O,O-bis(4,4'-dimethoxytriphenylmethyl)-2-O-(2-cyanoethyl)-N,N-diisopropylglycerolphosphoramidite, Figures 3 and 8). Each coupling doubles the number of hydroxyl groups available for the next coupling. The cycle can be repeated as many times as practical. Initial simplified molecular dynamics studies indicate similarity of the overall shape and dimension to the classical dendrimers. Up to 6th generation (64 hydroxyl groups) no changes in the synthesis cycle are necessary. Further coupling reaction increased amount of phosphoramidite used and prolonged coupling time. After propagation cycles, when desired number of hydroxyl groups is reached, aminomodifier is used (6-N-(4-monomethoxytriphenylmethyl)-1-O-(2-cyanoethyl)-N,N-diisopropyl-6-aminohexan-1-ol phosphoramidite, Figure 6.). This reagent is protected with a monomethoxytrityl group (4-monomethoxytriphenylmethyl, MMT), which is more appropriate for the N-protection, but provides similar quantitative assay of the coupling efficiency as the DMT group. The last coupling of aminomodifier can be followed by deprotection of the MMT group, and then the reaction column can be stored at low temperature and used as a stock for further reaction with Bolton-Hunter reagent (Figure 11).

SuperTracers with low NSBB. The use of SuperTracers may permit further diminishment of nonspecific biological background (NSBB). Second and third generation SuperTracers are essentially spherical in shape and very large. Thus, there is a mismatch between its size and the size of topological defects on the surface of the plastic. Furthermore, the forces imparted on the dendrimer by streaming washing liquid are very large, *i.e.*, stronger than the chemical forces of non-specific binding, and washing is therefore very efficient. However, these forces are lower than the strength of covalent bounding, biotin-avidin or antibody-epitope binding.

The NSB kinetics suggest a saturation phenomenon. There are only a limited number of NSB sites on the plastic - once these sites are filled no additional NSB will occur. We disclose the use of unlabeled dendrimers to fill and saturate the nonspecific binding sites before we add the radiolabeled SuperTracer. We also disclose the use of a mixture of unlabeled dendrimers of smaller generations as the carrier in our assay buffer.

Finally, we observed that a large part of the NSB is due to the interaction of streptavidin with the solid state support, *e.g.*, plastic of microtiter plates. We disclose a method which permits NSB diminishment by performing the biotin-streptavidin conjugation at low temperature followed by a very stringent wash more than five times. We also

documented a strong and *a priori* difficult to expect dependence of the NSB on the pH in which the radiolabeled streptavidin or streptavidin-SuperTracer interact with the biotinylated antibodies. Signal of a SIRMA in which the last step of labeling using ^{125}I-streptavidin is performed at a different pH. A strong signal increase is seen at pH =6.5. However, the conjugation of ^{125}I-streptavidin at lower pH leads to even larger increase of NSB. The result is that the best conditions for SIRMA is at a pH =7. Similar results have been achieved for different targets, *e.g.*, for the cytokines IL-6 and IL-12. This suggests that the influence of pH on the background is due to changes in the dynamics of the streptavidin interaction with the plastic of microtiter plates and bioanalytes. We disclose the use of an optimal pH, *e.g.*, pH = 7 in the case of microtiter plates, at the step when radiolabeled streptavidin or streptavidin/SuperTracer are used.

Direct quantitation of DNA using the SuperTracers. Direct DNA quantitation will become a necessary tool in assays both for genome incorporation tests as well as expression modulation tests using mRNA screening. In many implementations the tool of choice is polymerase chain reaction (PCR). In the case of *e.g.*, methylated DNA PCR fails and direct hybridization based methods should be developed. First method to be used with the phospate based SuperTracer are hybridization tests on the membranes: dot blot, and Southern blot. The first involves immobilization of the long DNA target on the membrane (positively charged) and hybridization of the labeled probe - usually labeled oligonucleotide. Southern blot involves separation step using electrophoresis, and subsequent transfer of the DNA onto the membrane (blotting). Again detection is achieved using labeled hybridization probe. Super tracer by introduction of multiple label attached through the linker arm to the 5'-end of the oligonucleotide provides strong signal without duplex destabilization. However, membranes introduce significant background due to non specific binding of the probe. This background becomes very pronounced when the quantitation is at the attomole level.

An alternative method that provides diminished background involves use of primary hybridization probe for the target DNA. This primary probe is immobilized on an appropriate support, preferably polystyrene plates or magnetic beads. Immobilization can be achieved either by using chemical methods or biotin-streptavidin binding. Hybridization of the target DNA in this case serves also as a purification step and allows using a mixture of DNA fragments *e.g.*, resulting from restriction enzyme digestion. Subsequently, labeled probe is used followed by detection/quantitation. This pseudo-sandwich assay uses

additional coincidence *i.e.*, the signal is obtained only when both probes are hybridized, which makes it equivalent to logic operator AND.

A third method that provides increased specificity is based on the format described above (capture of the target strand on an immobilized probe). The difference is in the use of a third probe, where target DNA, detection probe and auxiliary probe form a cruciform-type structure of three "branches". Preferably, the branch formed by the detection and auxiliary probes is designed to contain cleavable {Switch} structure, preferably restriction enzyme cleavage site. After hybridization of all probes, appropriate cleavage reagent, preferably restriction enzyme is used, and quantitation is done only on the material released from the solid support. This method introduces an additional proof-reading step that increases specificity of the whole assay.

Preferred implementation of {Switch}. The preferred method for dqDNA involves a place on the Double stranded DNA that can be cleaved in a controlled manner (environment). The preferred implementation uses double stranded DNA and a sequence recognized by an appropriate restriction enzyme as a {Switch}. This allows the implementation of a large family of switches, because there are a few hundred restriction enzymes. For example, one can use the family of restriction enzymes with the highest specificity, *i.e.*, those restriction enzymes which recognize the sixmer pattern of oligonucleotides. A particular implementation uses high specificity restriction enzymes selected from the following list:

Apa I [GGGCCC]; Asn I [ATTAAT]; BamH I [GGATCC]; Bcl [TGATCA];
Bgl II [AGATCT]; BspE I [TCCGGA]; BstB I [TTCGAA]; Cla I [ATCGAT];
Dra I [TTTAAA]; Eag I [CGGCCG]; EcoR I [GAATTC]; EcoR V [GATATC];
Fsp I [TGCGCA]; Hind III [AAGCTT]; Hpa I [GTTAAC]; Kpn I [GGTACC];
Kpn2 I [TCCGGA]; Mlu I [ACGCGT]; Msc I [TGGCCA]; Nar I [GGCGCC];
Nco I [CCATGG]; Nhe I [GCTAGC]; Nru I [TCGCGA]; Nsi I [ATGCAT];
PaeR7 I [CTCGAG]; Pst I [CTGCAG]; Pvu I [CGATCG]; Pvu II [CAGCTG];
Sac I [GAGCTC]; Sca I [AGTACT]; Sma I [CCCGGG]; SnaB I[TACGTA];
Spe I [ACTAGT]; Sph I [GCATGC]; Xba I [TCTAGA]; Xho I [CTCGAG];

wherein the restriction enzyme site consisting of a six nucleotide pattern is quoted within in the bracket. A particularly advantageous implementation of the invention uses restriction enzyme sites which contain only G and C bases, *i.e.*, nucleotide patterns which after complementation to double stranded DNA fragments have a high melting temperature. The said restriction enzymes are indicated in the above list by the underlined base sequences.

In this case the recognition site is a restriction enzyme site of the two stranded DNA used as {Switch/linker}. Actually, because of commercial availability of biotinylated antibodies it may be more practical to use the structure as follows :

$$\{\text{streptavidin}\} + \{DNA\}_1 + \{RES\}_i + (DNA)_2 + \{ST/DNA_{c2}\}$$

wherein $\{DNA\}_1$ is a two stranded DNA, $\{RES\}_i$ is a restriction enzyme site, and

$$\{DNA\}_2 = \{\text{two stranded DNA linker}\} + \{\text{single stranded DNA}\}$$

wherein the $\{ST/DNA_{c2}\}$ is a SuperTracer conjugated to a single stranded DNA the end of which is complementary to a single stranded DNA which is part of $\{DNA\}_2$. Note, that this construction permits us to operate with reagents which are not radioactive, and then perform the step of conjugating the radiolabeled conjugate the $\{ST/DNA_{c2}\}$ only after stringent wash.

The "three-probe" direct DNA quantitation using SuperTracers. The disclosed implementation of the "three-probe" DNA quantitation consists of a series of steps:

1) hybridize the target DNA on a solid surface using appropriate capture probe;
2) stringent wash;
3) hybridize detection probe with the target;
4) stringent wash;
5) hybridize auxiliary probe to the target and detection probe;
6) stringent wash;
7) adjust the buffer to restriction enzyme optimum, add enzyme;
8) incubate the digestion mixture;
9) collect supernatant; and
10) quantitate using MPD instrumentation.

Each of the washes in steps 2, 4 and 6 should be performed in optimal conditions, which may involve a change of temperature and/or pH. Furthermore, step 7, *i.e.*, adjustment of the buffer is necessary if sub-attomole sensitivity is required.

Antibody-Supertracer conjugation. SuperTracer can be attached to antibodies by using the innovative procedures described in the following. Amino groups can be attached to DNA molecule and can be utilized for derivatization with a number of available agents. Preferably the amino group is reacted with active carboxyl esters such as 4-nitrophenyl,

succinimidyl, sulfosuccinimidyl, or pentachlorophenyl. If anhydrous conditions can be appropriate it is possible to use a reaction with acyl or sulfonyl chlorides.

On the other hand synthetic oligonucleotides can be modified at the 5' end to incorporate reporter groups on an appropriate linker. Preferably the oligonucleotide contains an amino or mercapto group at the 5' end. It is possible to introduce other groups, but most preferable are those that are stable in deprotection conditions (*i.e.*, aqueous ammonia at elevated temperature). In the most preferable embodiment of the present invention, oligonucleotide is modified with a mercapto group at the 5' end and reacted with a maleimide group generated on the dendrimer surface by means of a reagent such as N-(4-maleimidobutyryloxy) succinimide ester. It is possible to achieve conjugation of an oligonucleotide to the dendrimer by using the following chemical reactions involving the amino group and the DNA:

* photoactivated reagents such as 4-azidosalicylic, 2-nitro-4- azidophenyl, 4-azidophenyl, and the like;
* active esters as described above;
* acyl and sulfonyl halides in anhydrous conditions as mentioned above;
* imidoesters to form amidine moiety;
* isothiocyanates to form thiourea moiety Sulfhydryl (mercapto) group on the DNA;
* maleimide to form a non-cleavable bond (mercaprtosuccinimidyl derivative);
* active disulfides such as 2-pyridyl to form a cleavable disulfide bond; and
* phosphorothioate group on the DNA can be alkylated with a variety of alkylating agents such as iodoacetic acid.

The sandwich immunoassay enhanced by the use of SuperTracers. The disclosed implementation of the 2-plex immunoassay consists of a series of steps:

1) capture the target on a solid surface using appropriate antibody Ab_1;
2) stringent wash;
3) conjugate biotinylated Ab_2 to the captured target;
4) stringent wash;
5) block using appropriate blockers, *e.g.*, the noniodinated starburst dendrimers;
6) stringent wash;
7) adjust pH to optimal value, typically pH = 7.0;
8) add radiolabeled SuperTracer with the appropriate streptavidin linker;
9) stringent wash; and

10) quantitate using MPD instrumentation.

Each of the washes in steps 2, 4 and 6 should be performed in optimal conditions, which may involve a change of temperature and/or pH. Furthermore, step 7, *i.e.*, adjustment of pH before the conjugation of SuperTracers to biotinylated Ab_2, is necessary if sub-attomole sensitivity is required.

The disclosed duplex immunoassay has considerable advantages over the classical sandwich immunoassay, because it allows considerable signal amplification. Typically, in a sandwich immunoassay a single reporter label *e.g.*, a fluorphore or a single radiolabel, is placed upon second Ab_2. In the disclosed assay a large number of labels are attached to each Ab_2. This leads to a 100 to 1,000 fold signal amplification. Thus, a shorter incubation time is required which usually considerably diminishes the NSB and tends to improve the signal/background ratio. We also stress the importance of performing sophisticated wash in step 9. The wash conditions may include higher temperature and cycling the pH from acidic to neutral to basic to neutral. Also, washing with an ultrasonic washer and "shock wave" washing was demonstrated to improve the signal to background ratio. Essentially, we tend to use "exponential wash" conditions, wherein the signal is considerably diminished (by a factor of five or more) but the non-specific background is almost eliminated (by a factor of hundred or more). The use of the large SuperTracer molecule permits to reach these "exponential wash" conditions. The force on the SuperTracer can be hundreds of times larger than the force on ^{125}I-avidin.

We established that a large part of the nonspecific background is due to the nonspecific binding of streptavidin to the solid support, *e.g.*, plastic of microtiter plates. Also, in many cell assays some biotin may be present leading to nonspecific biotin-streptavidin binding. Thus, the use of SuperTracers linked to a DNA probe may be an important tool in background rejection. The said single stranded DNA probe binds very specifically to their complementary DNA strand. Thus we disclose also the use of antibodies conjugated to single strand of DNA instead of biotinylated secondary antibodies. In the following we call such a hybrid the [Ab_2 + DNA] construct.

The preferred implementation of the 2-plet immunoassay using the [Ab_2 + DNA] construct consists of the following steps:

1) target capture on a solid-surface using an appropriate antibody Ab_1;
2) stringent wash;
3) conjugate the [Ab_2 + DNA] construct to the captured target;

4) stringent wash;

5) block using the appropriate blockers, *e.g.*, the noniodinated starburst dendrimer;

6) stringent wash;

7) adjust pH and temperature for optimal DNA hybridization;

8) add radiolabeled SuperTracer with the appropriate DNA linker(s);

9) stringent wash; and

10) quantite using MPD instrumentation.

There exist specific DNA probes which have a melting temperature below 40°C, *i.e.*, below the temperature of denaturation of proteins. Thus, the step of SuperTracer hybridization can be made more specific by temperature cycling around the melting temperature of the said specially designed DNA probe with appropriate washing. More specific hybridization can be achieved leading to elimination of this component of non-specific background. Our previous MPD enabled studies of the DNA hybridization background suggest that NSB of a few zeptomole is achievable.

The SuperTracer enhanced superfast immunoassay. Antibody binding is a very characteristic function of time. Typically, good binding probability, say above 90%, is achieved after quite a long period (longer than an hour). For short binding times, the binding probability is a linear function of time. The slope depends on the antibody quality and buffer conditions, *e.g.*, 37°C temperature increases the binding probability. It also depends on the format of the immunoassay, and usually the use of beads, *e.g.*, magnetic beads is favored for short assay times. For a short binding time (10 minutes), the binding probability may be as low as 10%. Thus, in the case of sandwich assays the probability that both antibodies will find and bind to their respective epitopes is very small, say a few percent. Therefore, the use of both very sensitive detectors, *e.g.*, MPD instrumentation and signal amplification, *e.g.*, the use of SuperTracers, are especially advantageous for implementing immunoassays shorter than 10 minutes. Our studies suggest that the non-specific background diminishes at least proportionally to the conjugation time. Actually, there is some evidence that S/B improves when the binding probability is smaller than 20%, *i.e.*, non-specific epitopes are of lower avidity than target epitopes.

We disclose the SuperTracer enhanced fast immunoassay using magnetic beads to improve the antibody-epitopes binding dynamics. However, similar fast immunoassays can be implemented using either plastic beads or microtiter format. The enabling *novum* is the use of supersensitive MPD instrumentation and signal amplification *via* SuperTracers to

allow very inefficient binding, *i.e.*, to permit very short conjugation time. The avidity of the biotin-streptavidin interaction is much higher than that of antibody-antigen binding and the conjugation time of the SuperTracer is not a rate limiting step of the disclosed immunoassay. Also, the magnetic beads removal time is shorter than the duration of stringent wash and has been selected as the preferred implementation of the SuperTracer enabled "fast immunoassay".

The time necessary to achieve a reproducible immunoassay is very dependent on the required sensitivity level. Thus, prior art immunoassays need about an hour to reach the 0.1 femtomole/ml level whereas "fast immunoassays" (shorter than 10 minutes) currently achieve only about 10 femtomole/ml sensitivity. The disclosed immunoassay is not only faster but also more sensitive; we designed an immunoassay with 50 attomole/ml sensitivity and a duration of less than 10 minutes.

The preferred implementation of the disclosed 2-plex fast immunoassay consists of the following steps:

1) capture the target on magnetic beads using an appropriate antibody Ab_1;
2) remove and wash magnetic beads;
3) conjugate the biotinylated Ab_2 to the captured target;
4) wash and block using the appropriate blockers, *e.g.*, the noniodinated starburst dendrimer;
5) add radiolabeled SuperTracer with the appropriate streptavidin linker;
6) wash; and
7) quantitate using MPD instrumentation.

The capture and conjugation steps are expected to take less than three minutes, each. The conjugation of SuperTracer is expected to take less than one minute. Each wash step is expected to take about 15 seconds. The read-out time may be as short as 30 seconds and all titration curves will be measured in parallel. Thus, a less than 10 minutes immunoassay seems feasible.

Other applications of SuperTracers with DNA linker. The above described SuperTracers with an attached DNA probe are very important innovative reagents for a plurality of applications in which signal amplification is used to enable the direct detection and quantitation of nucleic acids interactions with other biological macromolecules. These methods are enabled by both the use of super-sensitive MPD technique and the signal amplification by means of radiolabeled SuperTracer. Once more, the main challenge is the

rejection of non-specific biological background. The application of SuperTracers to direct detection of DNA/protein interaction is disclosed in a patent application titled "New methods for DNA/protein interaction".

EXAMPLE 1: Synthesis of 4th generation dendrimer on a 30-mer oligonucleotide probe.

Synthesis of the oligonucleotide hybridization probe was performed on the solid support using 0.2μmol synthesis column (1000Å, Long Chain Alkylamine Controlled Pore Glass, LCA CPG, Applied Biosystems) with 5'-O-dimethoxytriphenylmethyl-(4-N-benzoyl)-2'-deoxycytidine attached as a first building block. The synthesis was performed in an automatic DNA synthesizer (Applied Biosystems, 380B). Standard nucleoside phosphoramidites were purchased from Perkin Elmer (Applied Biosystems, MasterPiece, 500mg). Modified phosphoramidites were purchased from Clontech:

- linker reagent - (ω-O-(4,4'-dimethoxytriphenylmethyl)-1-O-(2-cyanoethyl)-N,N- diisopropyl tetraethylene glycol phosphoramidite 1 (100mg).
- forking reagent - (1,3-O,O-bis(4,4'-dimethoxytriphenylmethyl)-2-O-(2-cyanoethyl)- N,N-diisopropylglycerolphosphoramidite 2 (100mg).
- aminomodifier - (6-N-(4-monomethoxytriphenylmethyl)-1-O-(2-cyanoethyl)-N,N- diisopropyl-6-aminohexan-1-ol phosphoramidite 3 (100mg).

Each nucleoside phosphoramidite was dissolved in 3.0ml of anhydrous acetonitrile. Each modified phosphoramidite (1,2, and 3)was dissolved in 1.2ml of anhydrous acetonitrile.

After synthesis of 30-mer oligonucleotide, five couplings of the liner 1 were performed using standard synthesis cycle. Subsequently four coupling cycles of the forking reagent 2 were performed; third and fourth cycle being modified in order to provide increased delivery of the phosphoramidite to the synthesis column (double and quadruple, respectively), as the number of the hydroxyl groups available for coupling is doubled in each cycle. In the last step of the synthesis, aminomodifier 3 was used with quadruple delivery of the phosphoramidite at the coupling step. Modifications of the cycle were done by adding steps to the original cycle. In the present example the following additional steps were used: B+TET to column, TET to column, and WAIT. In each cycle following the synthesis of the oligonucleotide was monitored by means of dimethoxytrityl cation assay (DMT assay), which provides quantitative estimation of the coupling efficiency. Coupling efficiency for each of the linker 1 couplings was never less than 99.5%, for the forking reagent 2 varied

from 98% to 80% with overall yield of 60%, and the coupling of the aminomodifier 3 was above 99%. Aminomodifier coupling is less reliable, because different protecting group, namely monomethoxytriphenylmethyl (monomethoxytrityl, MMT), is used as more appropriate for protection of the primary amine, and the result is corrected for different extinction coefficient of the MMT cation.

Two other control syntheses were performed for the above example, namely unmodified oligonucleotide, and an oligonucleotide with one molecule of the aminomodifier 3 attached to the 5' terminus of the oligonucleotide. Three samples were coded as follows:

PDP5.4.1 - oligonucleotide 30-mer with 5 spacer molecules, 4th generation phosphate- based dendrimer, and 16 terminal amino groups at the 5' terminus.

PDP0.0.1 - oligonucleotide 30-mer without spacer arm and one aminomodifier molecule at the 5' terminus.

PDP - control unmodified oligonucleotide 30-mer.

Part of the solid support after the synthesis (2mg, with the product attached) were treated with gaseous ammonia for 10h as described by Boal *et al.* (1997), and eluted with 500μl of PBS buffer, pH 7.3. The aliquots were used for analysis, while the rest of the solid support was stored for further derivatization.

Each product was analyzed by HPLC in two-stage purification/analysis procedure: DMT-on, and DMT-off. HPLC was performed in 1%/min. linear gradient of acetonitrile in 0.1M triethylammonium acetate (TEAC) buffer, pH 7.2, starting with 0% RT HPLC column was used (Supelco, Supelcosil LC-318, 5μ 3.4mmx30cm). DMT-on chromatograms were produced.

Final efficiency of the synthesis calculated from the chromatogram was 60%, 90% and 90% for PDP5.4.1, PDP0.0.1, and PDP respectively.

Fractions containing target oligonucleotide were collected and lyophilized. Acetic acid (200 μl, 80% v/v) was added in order to remove 5'-DMT Group. After 10 min. the samples were evaporated to dryness and redissolved in TEAC HPLC buffer (200μl). Repeated HPLC analysis of the DMT-off samples in the same gradient showed a single peak of the product.

DMT-off chromatograms were produced.

Solid support containing PDP5.4.1 can be stored at -20°C for at least several months. Prior to radioiodination reaction, the support is deprotected with trichloroacetic acid (3% w/v in methylene chloride, 3ml), and washed with acetonitrile.

The texts of all publications including printed Patents mentioned herein are expressly incorporated by reference.

It is understood that various other modifications will be apparent to and can be readily made by those skilled in the art without departing from the scope and spirit of the present invention. Accordingly, it is not intended that the scope of the claims appended hereto be limited to the description set forth above but rather that the claims be construed as encompassing all of the features of patentable novelty which reside in the present invention, including all features which would be treated as equivalents thereof by those skilled in the art to which the invention pertains.

WHAT IS CLAIMED IS:

1. A bioreagent which is a SuperTracer for an analyte of interest which has been labeled, comprising:

a linker arm moiety;

at least one forking moiety provided on the linker arm moiety and having terminal branches; and

at least one reporter moiety provided on respective terminal branches of the at least one forking moiety,

wherein the bioreagent is effective to conjugate a predetermined number of labels;

wherein the bioreagent is effective to conjugate to biopolymers by means of the linker arm, and

wherein the bioreagent has a nonspecific biological background (NSBB) which is lower than about 10 attomole/well of a microtiter plate.

2. The bioreagent according to claim 1, wherein the labels are radiolabels.

3. The bioreagent according to claim 2, wherein the radiolabels comprise at least one multiphoton emitter, preferably at least one of an EC emitter and a positron/gamma emitter, which is compatible with multi photon detection instrumentation.

4. The bioreagent according to claim 3, wherein the radiolabels comprise at least one of ^{123}I and ^{125}I.

5. The bioreagent according to claim 2, wherein the SuperTracer which is radiolabeled is synthesized on a carrier, preferably a solid support as used in oligonucleotide chemistry, most preferably a controlled pore glass (CPG).

6. The bioreagent according to claim 2, wherein the SuperTracer which is radiolabeled is synthesized on a carrier after synthesizing at least one oligonucleotide on the carrier so that a covalently bound oligonucleotide-SuperTracer conjugate is produced.

7. The bioreagent according to claim 1, wherein the linker arm is a chain of one or more repeated building units.

8. The bioreagent according to claim 7, wherein the linker arm building unit is tetraethylene glycol phosphate.

9. The bioreagent according to claim 1, wherein the at least one forking moiety provides at least a doubling of the number of functional groups.

10. The bioreagent according to claim 9, wherein the functional groups are hydroxyl groups.

11. The bioreagent according to claim 9, wherein the at least one forking moiety is glycerol-2-O-phosphate.

12. The bioreagent according to claim 1, wherein the at least one reporter group is at least one of a primary or a secondary amino group on a linker, preferably on a carborn chain.

13. The bioreagent according to claim 12, wherein the at least one reporter group is used for labeling with a radiolabel.

14. The bioreagent according to claim 1, wherein the at least one reporter group is a oligonucleotide.

15. The bioreagent according to claim 14, wherein the oligonucleotide is used for labeling with a radiolabel.

16. The bioreagent according to claim 12, wherein the at least one reporter group is conjugated to biotin to provide a biotin conjugate.

17. The bioreagent according to claim 16, wherein the biotin conjugate is used for labeling with a radiolabel using one of avidin or streptavidin.

18. The bioreagent according to claim 1, wherein the at least one reporter moiety is at least one reporter group which is a sulfhydryl (mercapto) group.

19. The bioreagent according to claim 18, wherein the at least one reporter group is used for labeling with a radiolabel.

20. The bioreagent according to claim 1, wherein the linker arm has a terminal active group.

21. The bioreagent according to claim 20, wherein the terminal active group is selected from the group consisting of a primary amilo group, a secondary amino group, a sulfhydryl (mercapto) group, a carboxyl thereof, an ester thereof or halide thereof, a sulfonyl or ester thereof or halide thereof, an unsaturated ester, an amide, an imide, an ether, an aldehyde, and any group which is chemically conjugated.

22. The bioreagent according to claim 21, wherein the terminal active group is effective to conjugate with biomolecules selected from the group consisting of proteins, peptides, nucleic acids, oligonucleotides, lipids, polysaccharides, and carbohydrates, and any combination thereof.

23. The bioreagent according to claim 1, wherein the labels are radiolabels prepared by radioiodonation and are selected from the group consisting of ^{123}I, ^{124}I, and ^{125}I.

24. The bioreagent according to claim 23, wherein the radiolabels are radioiodionated moieties and are prepared by one of (a) reaction of one of the at least one reporter moiety, which is at least one reporter group, preferably an amino group, with at least one labeling agent, preferably a Bolton-Hunter reagent, or (b) another labeling reaction effective to label using a radioiodionated moiety.

25. The bioreagent according to claim 23, wherein the radioiodionation permits conjugation to a preselected number of radioiodine atoms on the carrier under anhydrous conditions.

26. The bioreagent according to claim 23, wherein the radioiodionation permits conjugation to a preselected number of radioiodine atoms on the carrier under aqueous conditions.

27. The bioreagent according to claim 23, wherein the radioiodionation permits conjugation to a preselected number of radioiodine atoms in solution after cleavage from the carrier under anhydrous conditions.

28. The bioreagent according to claim 23, wherein the radioiodionation permits conjugation to a preselected number of radioiodine atoms in solution after cleavage from the carrier under aqueous conditions.

29. The bioreagent according to claim 2, wherein the at least one forking moiety undergoes at least one coupling reaction and contains asymmetric blocking groups that can be selectively deprotected under different conditions, respectively.

30. The bioreagent according to claim 29, wherein the asymmetric blocking groups are dimethoxytriphenylmethyl (dimethoxytrityl, DMT) and levulinyl.

31. The bioreagent according to claim 30, wherein the at least one reporter moiety is at least one reporter groups and are positioned beneath the surface of the bioreagent which is a SuperTracer.

32. The bioreagent according to claim 31, wherein the at least one reporter groups are labeled with a radiolabel.

33. The bioreagent according to claim 2, wherein the at least one forking moiety is applied after initial forking and at least one linker arm coupling, resulting in initiation of a number of new spherical forking sites.

34. The bioreagent according to claim 33, wherein the at least one forking moiety has a number of couplings on the original linker arm which are called generations, and wherein the at least one forking moiety has a number of secondary forking layers which are called dimensions.

35. The bioreagent according to claim 34, wherein the number of generations ranges between 1 and 30, and the number of dimensions ranges between 1 and 10.

36. The bioreagent according to one of claims 22, 32 and 35, wherein the radiolabels and linkers, preferably biotin or DNA, are conjugated, and wherein at least one additional moiety is conjugated and is selected so as to be effective to diminish the nonspecific biological background (NSBB

37. The bioreagent according to claim 36, wherein the at least one additional moiety used to diminish the nonspecific biological background (NSBB) is polyethylene glycol (PEG).

38. An assay for direct DNA quantitation, comprising:

providing a bioreagent according to claim 1 which is a SuperTracer;

conjugating the bioreagent with a primary hybridization probe, which is preferably an oligonucleotide having a length ranging from 10 to 200 bases, to provide a conjugate; and

subjecting target DNA to the conjugate.

39. The assay according to claim 38, further comprising capturing the target DNA on a carrier selected from the group consisting of a membrane, a microtiter plate, a bead, and another solid carrier effective for biomolecule immobilization, wherein the target DNA is complimentary to the carrier.

40. The assay according to claim 39, wherein the target DNA is captured on the carrier by means of a complementary auxiliary probe, preferably an oligonucleotide having a length ranging from 10 to 200 bases, and wherein the complementary auxiliary probe is one of covalently or non-covalently attached to the carrier.

41. The assay according to claim 40, further comprising detecting the DNA by a technique effective to measure the conjugate of the SuperTracer and the primary hybridization probe.

42. The assay according to claim 41, further comprising conjugating the bioreagent with a secondary hybridization probe, which is preferably an oligonucleotide having a length ranging from 10 to 200 bases

43. The assay according to claim 42, wherein the primary and secondary hybridization probes have part of a sequence which is complementary to that of the target DNA and have another part of a sequence which allows hybridization of the primary and secondary hybridization probes to one another.

44. A bioreagent having a structure:

$$\{linker\}_1 + \{switch\}_i + \{linker\}_2 + SuperTracer,$$

wherein $\{linker\}_1$, $\{linker\}_2$ and SuperTracer are polymers.

45. The bioreagent according to claim 44, wherein $\{switch\}_i$ is activated under well-defined, preselected experimental conditions effective therefor.

46. The bioreagent according to one of claims 43 and 44, wherein $\{switch\}_i$ is composed of respective parts of the sequences of the primary hybridization probe and the secondary hybridization probe that are complementary to each other.

47. The bioreagent according to claim 46, wherein $\{switch\}_i$ contains at least one restriction site and can be cleaved under action of a specific restriction enzyme.

48. The bioreagent according to claim 44, wherein $\{switch\}_i$ is one of a polynucleotide or a DNA molecule which is chemically modified in such a way that it contains a number of amino acids, forming a DNA-peptide conjugate, which are cleaved under action of an enzyme that selectively recognizes and cleaves short peptide sequences and is one of a protease or a peptidase.

49. The bioreagent according to claim 44, wherein $\{switch\}_i$ is one of a polynucleotide or DNA molecule which is a chemically modified oligonucleotide fragment to which is incorporated one of a monosaccharide or an oligosaccharide in a preselected part of the molecule and wherein the monosaccharide or the oligosaccharide incorporated into $\{switch\}_i$ are cleaved by glycosidases which recognize and hydrolize specific glycosidic bonds of conjugated saccharides.

50. The bioreagent according to claim 44, wherein $\{switch\}_i$ is cleaved at a specific site, and wherein the cleavage is effected by the change of external thermodynamic parameters, preferably one of temperature or pH.

51. The bioreagent according to claim 50, wherein $\{switch\}_i$ is cleaved at a specific site by increasing temperature thereof, and wherein $\{switch\}_i$ consists of a DNA

linker constructed to contain short stretches of overlapping sequences which function as sticky ends and which are effective to provide strictly defined thermodynamic characteristics, preferably a steep transition in the melting curve, and melting point that would be released thermally in conditions that would not be detrimental either for the restriction enzyme activity or antibody binding.

52. The bioreagent according to claim 50, wherein $\{switch\}_i$ is cleaved at a specific site by decreasing pH thereof, preferably to a low pH ranging from 4 to 7, and is used for selective hydrolysis of chemical bonds that could be incorporated into linkages of the conjugate by use of carboxyl amides including some peptide bonds.

53. The bioreagent according to claim 50, wherein $\{switch\}_i$ is cleaved at a specific site by decreasing pH thereof to a low pH ranging from 4 to 7, and is used for selective hydrolysis of chemical bonds that could be incorporated into linkages of the conjugate by use of acid sensitive ethers, preferably triphenylmethyl ethers.

54. The bioreagent according to claim 50, wherein $\{switch\}_i$ is cleaved by decreasing pH thereof to a low pH ranging from 4 to7, and is used for selective hydrolysis of chemical bonds that could be incorporated into linkages of the conjugate by use of glycosidic bonds with a variety of saccharides, preferably sialic acid.

55. The bioreagent according to claim 50, wherein $\{switch\}_i$ is cleaved by decreasing pH thereof to a low pH ranging from 4 to 7, and is used for selective hydrolysis of chemical bonds that could be incorporated into linkages of the conjugate by use of acetals.

56. The bioreagent according to claim 50, wherein $\{switch\}_i$ is cleaved at a specific site by increasing pH thereof which causes one of (a) dissociation of hydrogen bonded counterparts, preferably DNA hybridization, or (b) a chemical reaction, preferably ester hydrolysis.

57. The bioreagent according to claim 50, wherein $\{switch\}_i$ is cleaved by increasing pH thereof to a high pH ranging from 7 to 10, and is used for cleavage of a site which was at least one of (a) engineered for cleavage at a specific pH and (b) used as a trigger to subsequent reactions.

58. The bioreagent according to claim 50, wherein the cleavage is triggered by the change of external thermodynamic parameters, and wherein the trigger is hydrolysis of carboxylic ester which releases the aromatic ring from the phosphate *via* two sequential nucleophilic substitutions on a phosphorus atom.

59. The bioreagent according to claim 50, wherein $\{switch\}_i$ is cleaved at a specific site, and wherein the cleavage uses disulfide bond chemistry in which the disulfide bond is selectively cleaved with one of DTT (dithiothreitol) or another mercaptane, preferably where the disulfide bond is used between an antibody and a 3' end of a oligonucleotide.

60. The bioreagent according to claim 50, wherein {switch} is cleaved at a specific site, wherein the cleavage uses siloxane linkage chemistry in which the siloxane linkage is removable by a fluoride ion $F^{\ddagger}$ of at least one water soluble salt, and wherein reaction in water is slow and provides very mild conditions.

61. The bioreagent according to claim 60, wherein the siloxane linkage is used in anhydrous conditions to accelerate cleavage.

62. The bioreagent according to claim 44, wherein $\{switch\}_i$ is cleaved at a specific site, wherein the cleavage uses alkene (double bonds) chemistry in which the double bond is selectively oxidized in a metal catalyzed reaction leading to cleavage of the bond, and wherein the metal catalyzed reaction preferably employs OsO4.

63. The bioreagent according to claim 44, wherein $\{switch\}_i$ is cleaved at a specific site, and wherein the cleavage uses Cis-diols (viconal diols with cis arrangement) chemistry in which the Cis-diols are selectively cleaved by periodates (IO_4) to break the carbon-carbon bond and produce two aldehyde functions.

64. The bioreagent according to claim 44, wherein $\{switch\}_i$ is cleaved at a specific site, and wherein the cleavage uses photolysis chemistry.

65. The bioreagent according to claim 44, further comprising an activator which is at least one restriction enzyme, and wherein $\{switch\}_i$ is a duplex DNA with a preselected sequence containing a restriction site.

66. The bioreagent according to claim 65, wherein the at least one restriction enzyme is a high specificity restriction enzyme selected from the group consisting of:

Apa I [GGGCCC]; Asn I [ATTAAT]; BamH I [GGATCC]; Bcl I [TGATCA]; Bgl II [AGATCT]; BspE I [TCCGGA]; BstB I [TTCGAA]; Cla I [ATCGAT]; Dra I [TTTAAA]; Eag I [CGGCCG]; EcoR I [GAATTC]; EcoR V [GATATC]; Fsp I [TGCGCA]; Hind III [AAGCTT]; Hpa I [GTTAAC]; Kpn I [GGTACC]; Kpn2 I [TCCGGA]; Mlu I [ACGCGT]; Msc I [TGGCCA]; Nar I [GGCGCC]; Nco I [CCATGG]; Nhe I [GCTAGC]; Nru I [TCGCGA]; Nsi I [ATGCAT]; PaeR7 I [CTCGAG]; Pst I [CTGCAG]; Pvu I [CGATCG]; Pvu II [CAGCTG]; Sac I

[GAGCTC]; Sca I [AGTACT]; Sma I [CCCGGG]; SnaB I[TACGTA]; Spe I [ACTAGT]; Sph I [GCATGC]; Xba I [TCTAGA]; Xho I [CTCGAG],

wherein the at least one restriction enzyme site consists of a six nucleotides pattern and is set out in respective brackets.

67. The bioreagent according to claim 66, wherein the at least one restriction enzyme site consists of only G and C, and has a nucleotides pattern which, after complementation to double stranded DNA fragments, has a high melting temperature; and wherein the at least one restriction enzyme is: Apa I [GGGCCC]; Eag I [CGGCCG]; Nar I [GGCGCC]; and Sma I [CCCGGG].

68. A method for improving immunoassay sensitivity to improve the limits of detection (LOD), comprising at least one of:

using a plurality of secondary antibodies in parallel to increase signal;

using a SuperTracer to amplify signal;

using a multi photon detection (MPD) instrumentation to quantitate signals at sub-attomole levels;

using a technique effective to diminish non-specific biological background (NSBB), preferably an "exponential" wash; and

using a releasable {Switch} to permit independent quantitation of remaining non-specific biological background (NSBB).

69. An immunoassay having improved limits of detection using a SuperTracer with an oligonucleide linker and which comprises:

a) capturing a target on a solid-surface using an antibody Ab_1 which is effective therefor;

b) stringent washing;

c) conjugatng to the captured target of [Ab_2 + DNA] construct;

d) stringent washing;

e) blocking using a blocker effective therefor, preferably a noniodinated SuperTracer;

f) stringent washing;

g) adjusting at least one of pH and temperature to levels effective for DNA hybridization;

h) applying a radiolabeled SuperTracer with an effective DNA linker(s);

i) stringent washing; and

j) quantitating using multi photon detection (MPD) instrumentation.

70. An immunoassay employing a bioreagent according to claim 46, comprising the steps of:

a) capturing a target on a solid-surface using an antibody Ab_1 which is effective therefor;

b) stringent washing;

c) blocking using a blocker effective therefor, preferably a noniodinated SuperTracer;

d) stringent washing;

e) conjugating to captured the target of a plurality of constructs;

f) stringent washing;

g) adjusting pH;

h) conjugating a radiolabeled SuperTracer to all [Ab_i + {Switch/linker}] constructs;

i) stringent washing;

j) quantitating with multi photon detection (MPD) instrumentation to measure signal and background;

k) activating a switch under conditions effective therefor;

l) stringent washing;

m) quantitation with multi photon detection (MPD) instrumentation to measure background; and

n) numerically subtracting signal and background from background to obtain a result.

71. An immunoassay employing a bioreagent according to claim 44, comprising the steps of:

a) capturing a target using one of a monoclonal or a polyclonal antibody Ab_1 effective therefor and attached to the solid-surface by a [{linker}$_1$ + {Switch}$_1$ +{linker}$_2$} construct effective therefor;

b) stringent washing;

c) blocking using at least one blocker effective therefor, preferably a noniodinated SuperTracer;

d) stringent washing;

e) conjugating to the captured target a plurality of constructs;

f) stringent washing;

g) adjusting pH;

h) conjugating a radiolabeled SuperTracer to all [Ab_i + $\{linker\}_1$ +$\{Switch\}_2$ +$\{linker\}_2$] constructs;

i) stringent washing;

j) quantitating with multi photon detection (MPD) instrumentation to measure signal and background;

k) activating the $\{Switch\}_2$ under conditions effective therefor;

l) stringent washing;

m) quantitating with multi photon detection (MPD) instrumentation to measure a sum of backgrounds;

n) activating the $\{Switch\}_1$ under conditions effective therefor;

o) stringent washing;

p) quantitating with multi photon detection (MPD) instrumentation to measure background due to non-specific binding to solid state; and

r) numerically subtracting the signal and background from the sum of backgrounds to obtain a result.

72. A kit of bioreagents having structures as follows:

$\{linker\}_1$ + $\{switch\}_i$ + $\{linker\}_2$ + SuperTracer,

wherein $\{linker\}_1$, $\{linker\}_2$ and SuperTracer are polymers and are always the same constituent, respectively, wherein only $\{switch\}_i$ is a variable constituent, and wherein the linkers can be implemented in a plurality of ways which include either as a chain of polypeptides or as an oligonucleotides.

73. The kit of bioreagents according to claim 72, wherein $\{switch\}_i$ is activated under well-defined, preselected experimental conditions effective therefor.

74. A bioreagent with high affinity for an analyte of interest, comprising:

a linker arm terminal moiety, R_1, which has an affinity for the analyte of interest;

a linker arm which is a polymer having a chain length effective to mitigate steric interference between R_1 and a forking moiety;

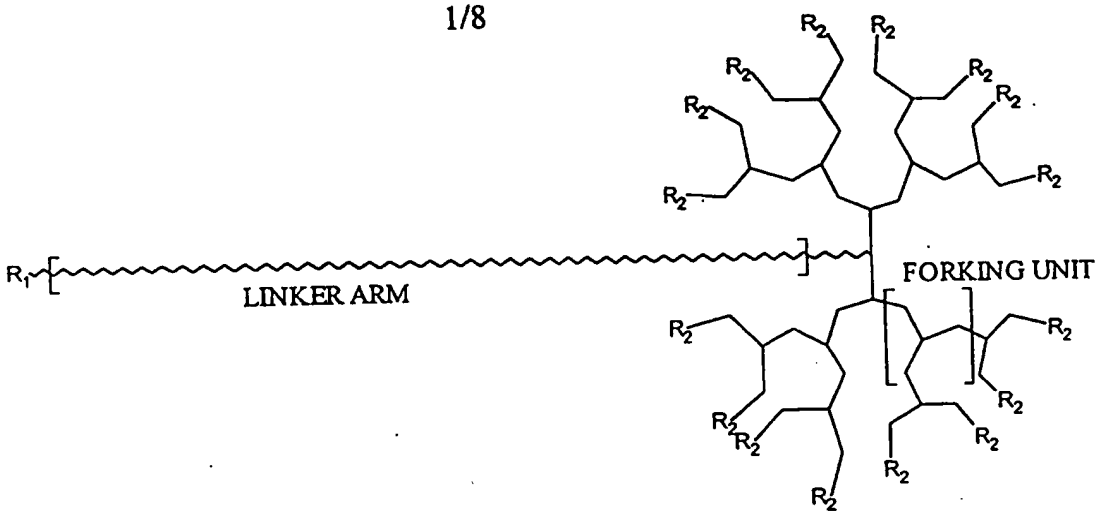
a forking moiety comprised of a substance having a spherical branched polymer structure including terminal functional groups; and

at least one reporter moiety, R_2, provided on respective terminal functional groups of the forking moiety.

75. The bioreagent according to claim 74, wherein the forking moiety has a first dimension with x forks, and second dendrimer dimension having y forks, and the reporter moiety is present in a n umber up to a number 2^{x+y}, where x is the number of forks in branches of the first dendrimer dimension and y is the number of forks in branches of the second dendrimer dimension.

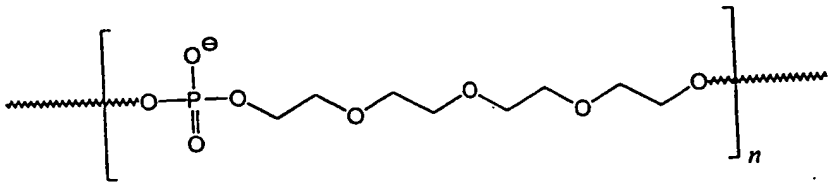
76. The bioreagent according to claim 75, wherein the forking unit comprises at least one forking unit linker arm and the forking unit comprises at least two spherical branched polymeric structures.

77. The bioreagent according to claim 74, wherein the number of terminal functional groups ranges between 16 and 128.



R_1 - linker arm terminal group
R_2 - reporter groups

Fig. 1



n = 1, 2, 3, ...

Fig. 2

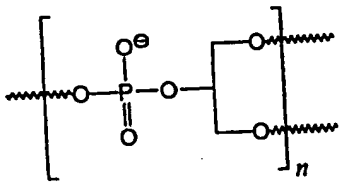


Fig. 3

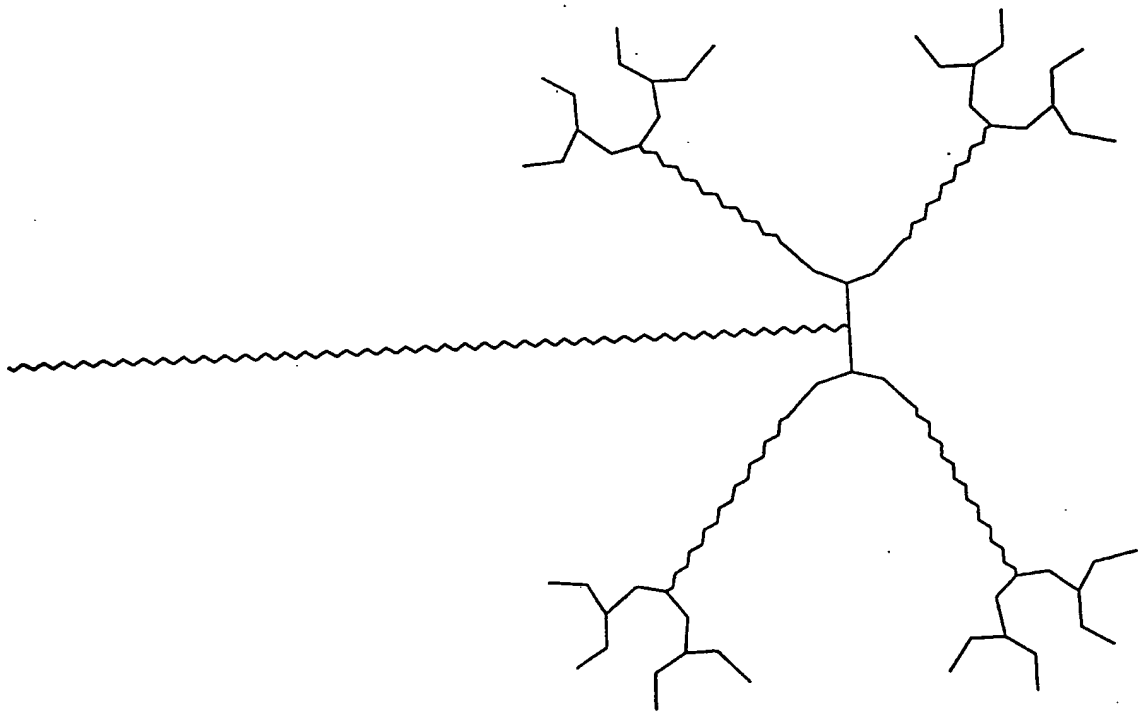


Fig. 4

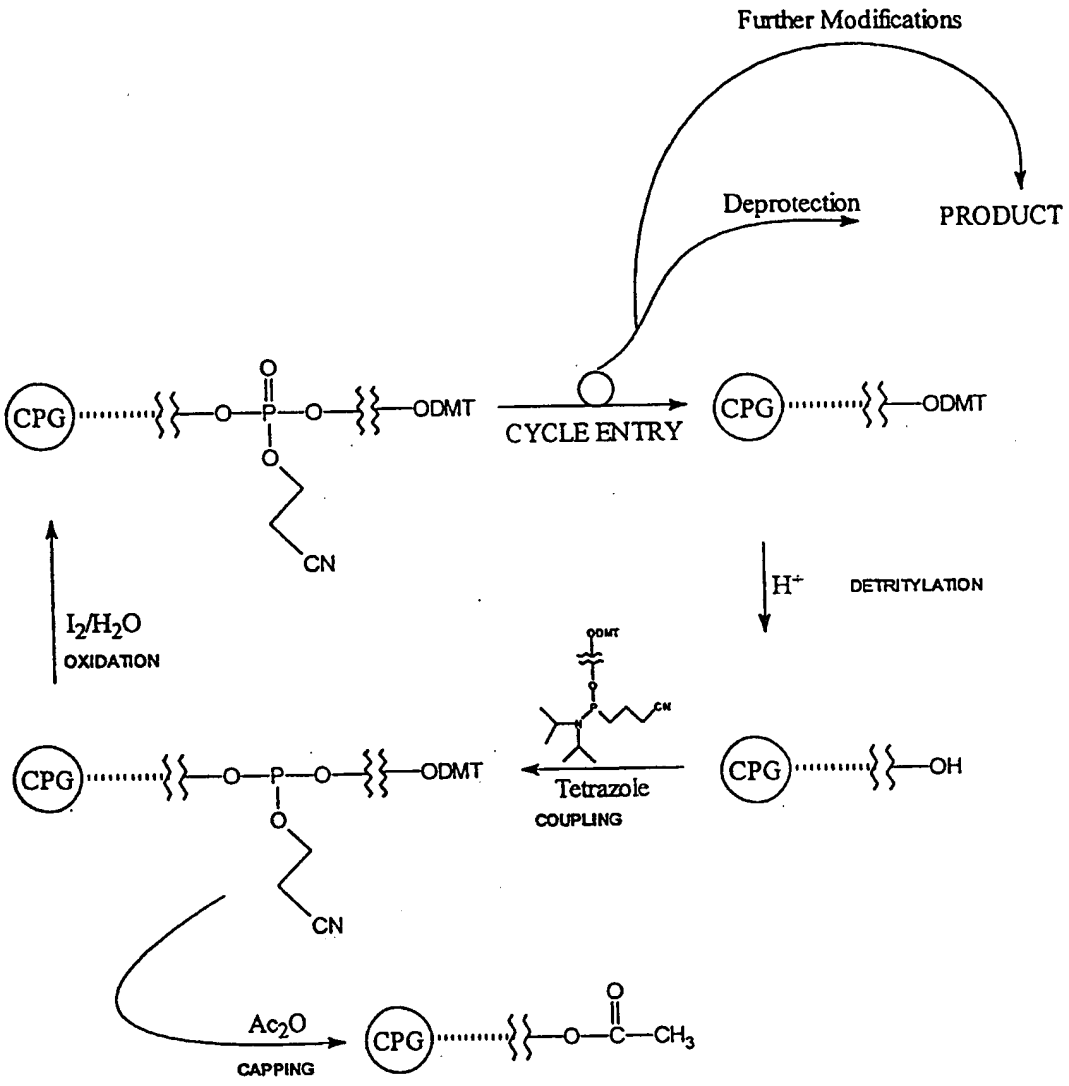

Further Modifications
Deprotection
PRODUCT
CPG
ODMT
CYCLE ENTRY
CN
I_2/H_2O
OXIDATION
H^+
DETRITYLATION
Tetrazole
COUPLING
OH
Ac_2O
CAPPING
CH_3

Fig. 5

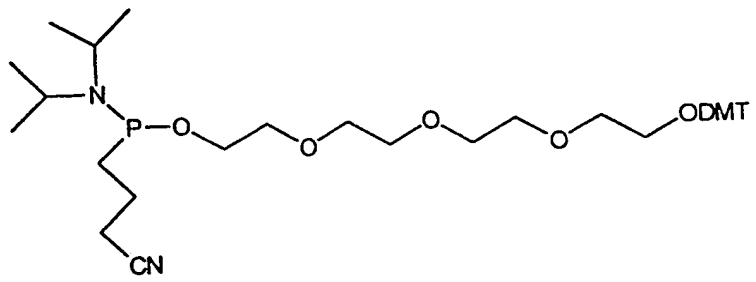


Fig. 6

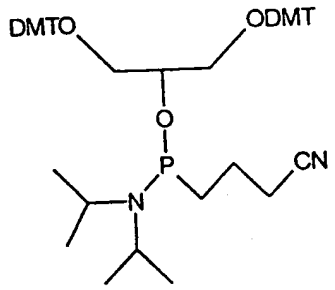


Fig. 8

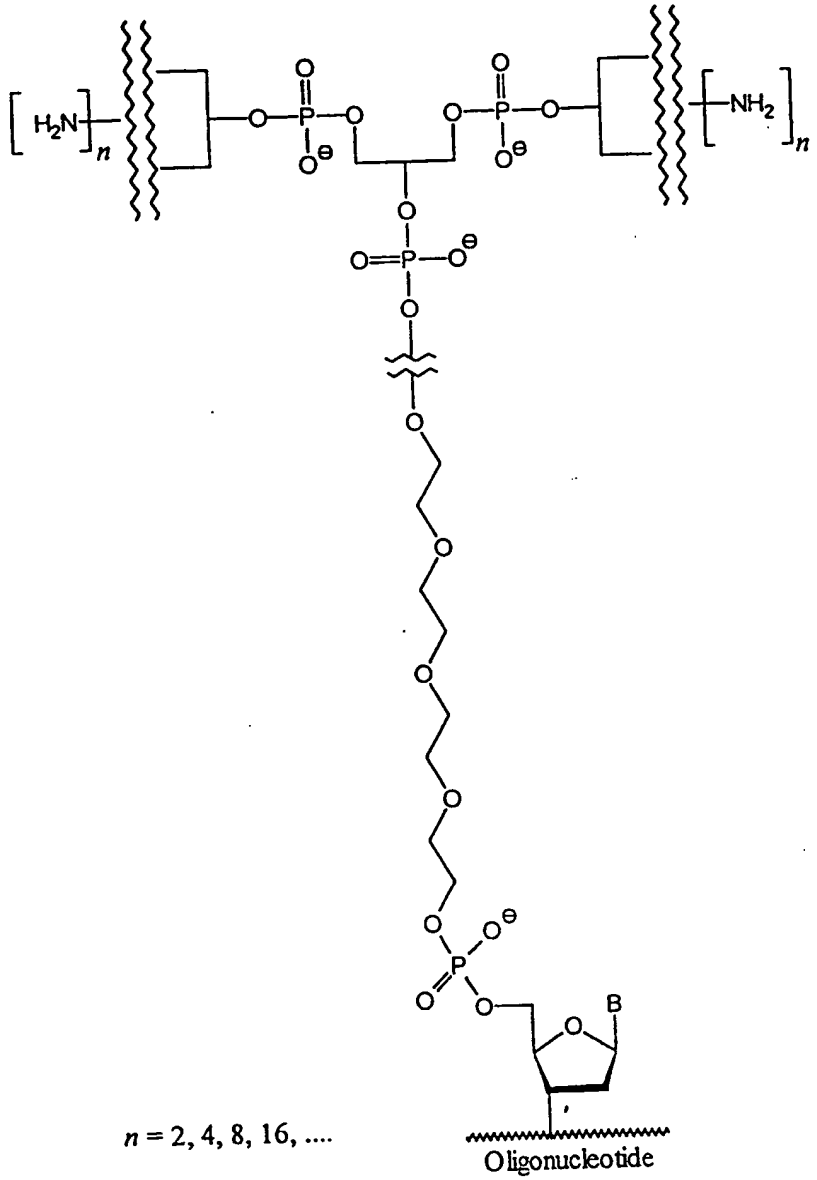


Fig. 7

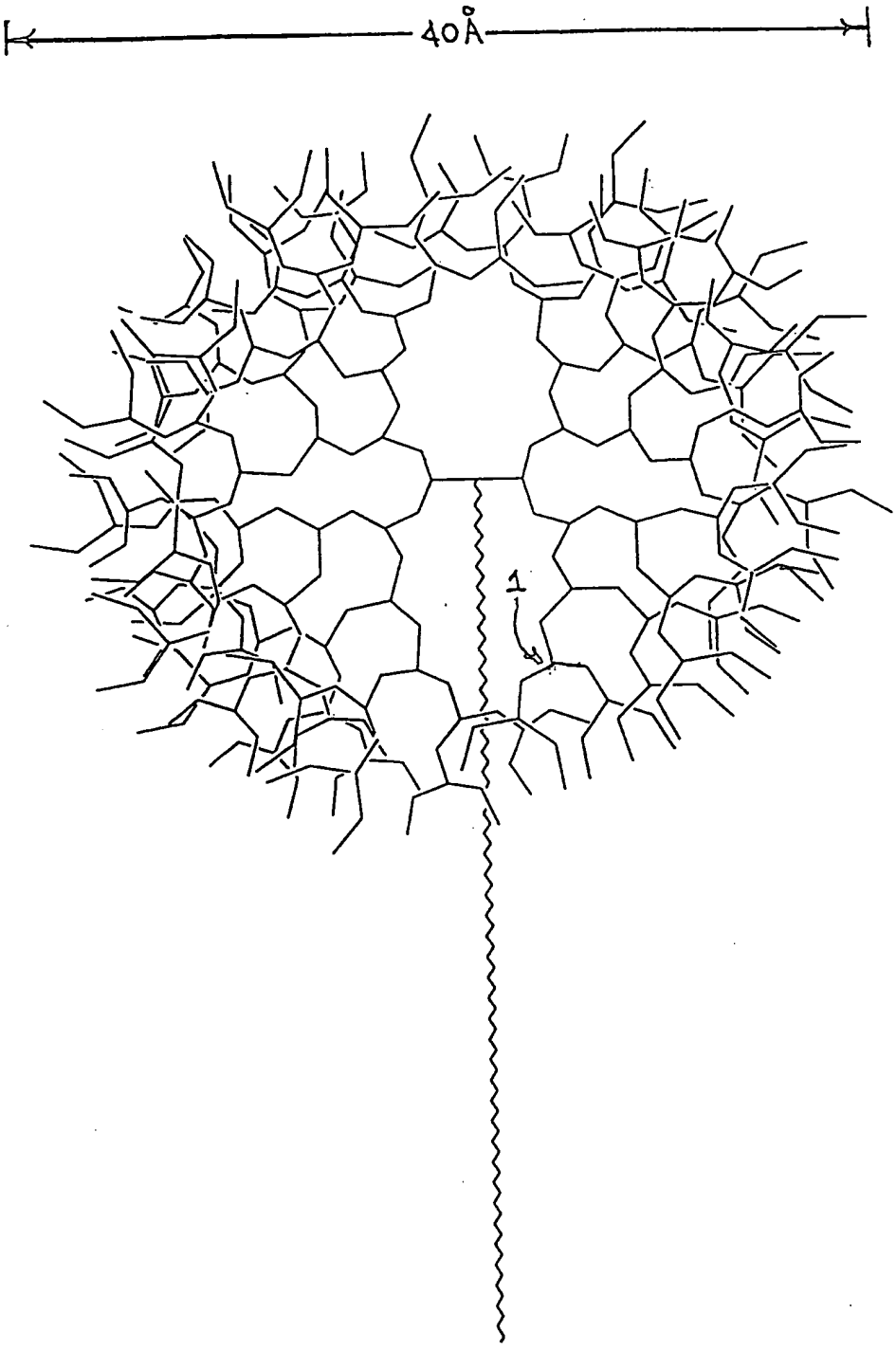


Fig. 9

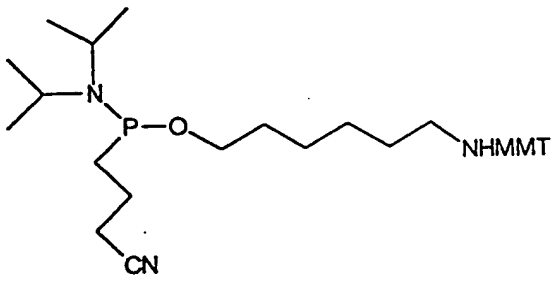

N
P
O
NHMMT
CN

Fig. 10

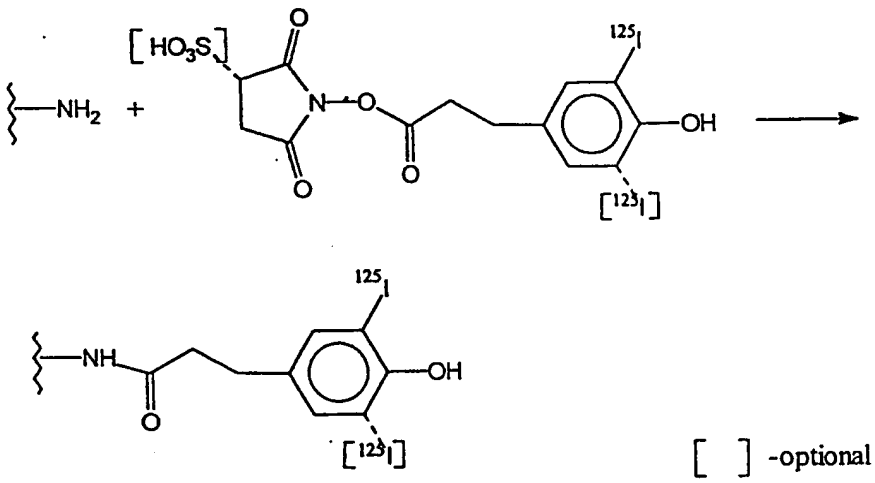

[HO3S]
O
NH2 +
N
O
O
O
125I
OH
[123I]
125I
NH
O
OH
[123I]
[ ] -optional

Fig. 11

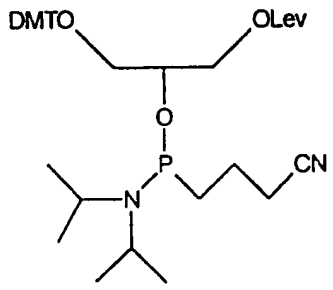


Fig. 12